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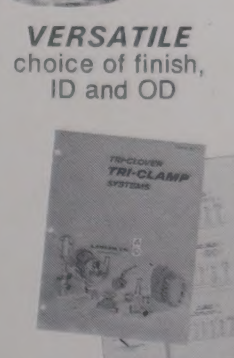

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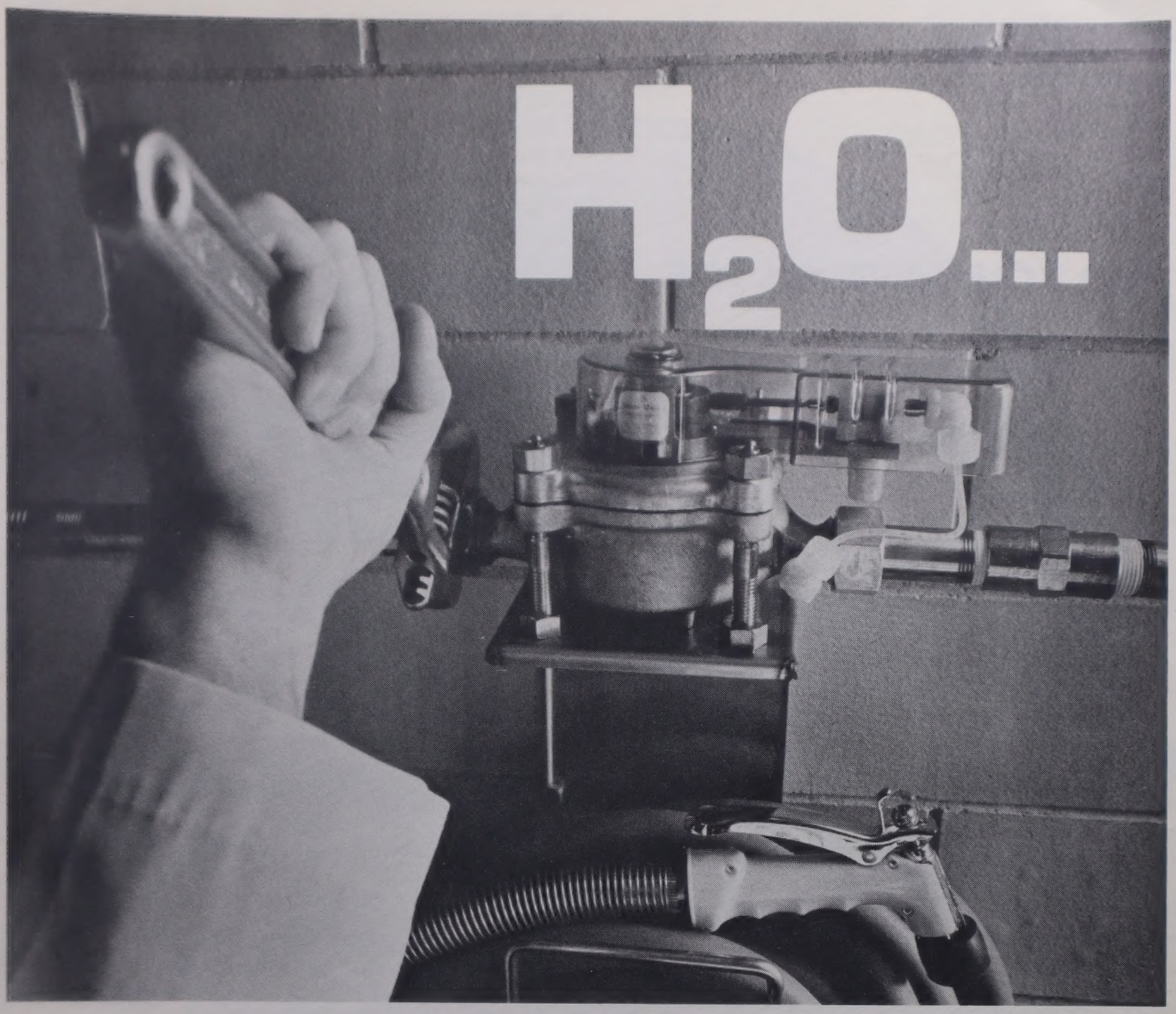

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Effect of Organochlorine Pesticides on Growth and Biological Activities of Bacterial Species Important to the Dairy Food Industry

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ABSTRACT

Three growth patterns (no effect, slight inhibition, or complete inhibition) were observed when bacterial species common to the dairy-food industry were grown in media containing 50 or 100 ppm DDT, dieldrin, or endrin. The pattern obtained appeared to depend on species and type and concentration of pesticide. All pesticides studied had a greater inhibitory effect on gram positive species than they had on gram negative species when grown in broth. Acid production by lactic acid bacteria was inhibited in broth plus 5 ppm chlordane or heptachlor but unaffected in skimmilk plus up to 100 ppm of these pesticides. Generation times for gram negative species grown in broth plus 10 ppm chlordane or heptachlor were similar to those obtained in controls. Growth of gram positive species was inhibited in broth plus 10 ppm of these pesticides but unaffected in skimmilk containing similar pesticide concentrations. Generation times for several gram negative species were increased by 10 ppm heptachlor in skimmilk.

Concern has increased during the last few years over the potential harmful effects that organochlorine pesticides might have on the environment due to their long-term persistence and to their resistance to degradation. These pesticides could enter the human food chain and affect growth and biological activity of microorganisms used to manufacture dairy-food products. In addition, inhibition of desirable microorganisms could create conditions which favor growth of pathogenic or spoilage organisms.

Published information does not agree on the effects that pesticides have on the biological activity of microorganisms. Some results indicate no effect on growth in milk containing up to 100 ppm of various pesticides (*8, 9, 12*), while other studies claim that pesticides inhibit growth (*2, 4, 7, 11*). Studies in this laboratory indicate that growth of selected bacteria in broth is inhibited by pesticide concentrations ranging from 3 to 100 ppm but unaffected when grown in milk containing similar pesticide concentrations (*4, 10, 11*).

This study was undertaken to determine: (*a*) the effect of DDT, dieldrin, endrin, chloradane and heptachlor on the growth and activity of selected bacterial species in broth and/or milk media; (*b*) the effect of chlordane and heptachlor on rate and amount of acid production by selected lactic acid bacteria grown in broth and skimmilk media; and (*c*) the effect of chlordane and heptachlor on the generation times of selected bacteria grown in broth and skimmilk media.

MATERIALS AND METHODS

Cultures

Bacterial species used in this study were obtained from the stock culture collection of the Food Science Section, Department of Animal Sciences, University of Kentucky. Species used and their optimum growth temperatures are listed in Table 1. In addition, commercial mixed strain starter cultures used in one phase of the study were obtained from two commercial culture companies.

Tomato juice broth (TJB) and Elliker broth (EB) were used for growth and maintenance of species belonging to the genera *Streptococcus, Leuconostoc, Pediococcus,* and *Lactobacillus.* Trypticase soy broth (TSB) and nutrient broth (NB) were used to grow and maintain the remaining species. The optimum growth temperature for each individual species (*3*) was used for incubation in this study.

Pesticides

Pesticides used in this study, along with their purity, chemical name and source are listed in Table 2.

Each pesticide was made up in a stock solution which contained 15 mg/ml absolute ethanol (1.0 μg/ml = 1 part per million), so that final concentrations of between 5 and 100 ppm could be obtained by adding no more than 2.0 ml of solution to 100 to 500 ml of medium. Pesticides were added immediately before the medium was inoculated with the test microorganisms.

Growth and maintenance

A 15 to 18 h actively growing broth culture was used to inoculate 100 ml of sterile medium containing the desired pesticide concentration. The culture was transferred daily for 3 days before 10^4 to 10^5 cells per ml of test medium were added.

Controls (no pesticides) were inoculated, incubated and analyzed under the same conditions as media containing 50 and 100 ppm pesticide. All cultures were incubated at their optimum growth temperature for up to 72 h.

The effect that the pesticides had on bacterial growth was determined by doing plate counts according to *Standard Methods* (*1*), except that tomato juice agar was used to enumerate lactic acid bacteria and trypticase soy agar was used for all other species. Depending on the optimum growth temperature for the test species, plates were incubated for up to 72 h. Enumerations were made by plating an aliquot of sample every 2 to 4 h during the initial 24 h of incubation. Additional counts were made every 24 h until the end of a trial, which generally lasted 72 h.

Growth in broth media containing between 5 and 20 ppm chlordane or heptachlor was determined by measuring the change in turbidity using a Klett-Summerson Photoelectric Colorimeter equipped with a 400-465 mμ filter.

Media plus pesticide were inoculated at a concentration of 10^5 cells/ml from a 15 to 18 h actively growing culture, incubated, and Klett values determined every 2 h during the initial 12 h and then at various times during the next 60 h. Controls were inoculated, incubated, and analyzed under the same conditions as media plus

TABLE 1. *Incubation temperature of bacterial species used in this study and effect on their growth in broth containing 10 ppm chlordane and heptachlor*

Species	Incubation temperature (C)	Pesticide (10 ppm) Chlordane	Heptachlor
Alcaligenes faecalis	37	–[a]	–
Bacillus cereus	30	+[b]	+
Bacillus cereus subsp. *mycoides*	30	+	+
Bacillus coagulans	45	+	+
Bacillus polymyxa	30	+	+
Bacillus stearothermophilus	55	+	–
Bacillus subtilis	30	+	–
Brevibacterium linens	30	NR	NR
Citrobacter freundii	37	NR	NR
Enterobacter aerogenes	30	NR	NR
Escherichia coli	37	–	–
Lactobacillus acidophilus	37	+	+
Lactobacillus brevis	30	+	+
Lactobacillus bulgaricus	37	+	+
Lactobacillus casei	37	+	+
Lactobacillus delbrueckii	37	+	+
Lactobacillus helveticus	37	+	+
Lactobacillus lactis	37	+	+
Lactobacillus leichmannii	37	+	+
Lactobacillus viridescens	37	+	+
Leuconostoc dextranicum	26	+	+
Leuconostoc mesenteroides	26	+	+
Microbacterium lacticum	26	+	+
Micrococcus luteus	26	NR	NR
Pediococcus cerevisiae	26	+	+
Proteus vulgaris	37	–	–
Pseudomonas aeruginosa	37	+	–
Pseudomonas fluorescens	26	–	–
Pseudomonas fragi	26	–	–
Salmonella typhimurium	37	NR	NR
Sarcina lutea	30	+	+
Serratia marcescens	26	NR	NR
Staphylococcus aureus	37	+	–
Staphylococcus epidermidis	37	NR	NR
Streptococcus bovis	37	+	+
Streptococcus cremoris	26	+	+
Streptococcus faecalis	37	+	+
Streptococcus faecium	37	+	+
Streptococcus lactis	37	+	+
Streptococcus pyogenes	37	+	+

[a] – = Growth not inhibited
[b] + = Growth completely inhibited
NR = Not run

pesticide. Flasks containing 0 (controls) and 5 ppm heptachlor or chlordane in either EB enriched with 5% lactose or SM enriched with 5 g yeast extract/1 were inoculated with 1% of actively growing lactic acid bacterial species or mixed strain commercial culture to determine the effect of pesticide on acid production. Flasks were incubated at optimum temperatures for the test species, and acidities (*6*) were determined at time of inoculation and then every 2 h for 12 h and again at 24 h.

Generation times were determined by inoculating flasks containing trypticase soy broth or skimmilk and either 0 (control) or 10 ppm chlordane or heptachlor, with 1% actively growing cultures and determining counts (*1*) every 2 h during the first 12 h of incubation. Generation times were calculated (*5*) from these data.

RESULTS AND DISCUSSION

Effect of DDT, dieldrin and endrin on bacterial growth

Three types of growth were obtained when nine bacterial species were grown in liquid media containing up to 100 ppm of DDT, dieldrin, or endrin. Growth was categorized as not affected, slightly inhibited, or completely inhibited by these pesticides; and the results appeared to depend on bacterial species, pesticide, and concentration of pesticide. The effects of the three pesticides on the nine bacteria studied are shown in Table 3. The effects are further illustrated in Figures 1, 2, and 3.

TABLE 3. *Effect of 50 and 100 ppm DDT, dieldrin and endrin on the growth of nine bacteria in broth after 24 h*[a]

Species	Pesticide: DDT 50	DDT 100	Dieldrin 50	Dieldrin 100	Endrin 50	Endrin 100
	Concentration (ppm)					
Escherichia coli	0	0	0	0	0	0
Serratia marcescens	0	0	0	0	0	0
Lactobacillus lactis	2.2	2.1	0.7	0.8	1.0	1.0
Micrococcus luteus	2.8	2.2	1.8	—	1.2	1.5
Pseudomonas fluorescens	1.6	1.9	0.2	0.5	1.5	0.9
Streptococcus lactis	3.4	3.3	0.9	0.9	0.3	0.3
Bacillus cereus	0	0	5.7	5.7	0.5	0
Staphylococcus aureus	2.3	1.4	—	5.8	0	0
Streptococcus faecium	1.7	1.8	0	0	0	0.5

[a] = Plate counts ($\log_{10}$) in controls minus plate counts ($\log_{10}$) in 50 or 100 ppm pesticide containing broth.

TABLE 2. *Chemical name. purity and source of the pesticides used in this study*

Pesticide	Purity	Chemical name	Source
DDT	Technical 77.2%	1,1,1-trichloro-2,2-*bis* (*p*-chlorophenyl) ethane	City Chemical Corp., New York, N. Y.
Heptachlor	Technical 72%	1,4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene	City Chemical Corp., New York, N.Y.
Endrin	99%	1,2,3,4,10,10-hexachloro-6,7-*epoxy*-1,4,4a,5,6,7,8a-octahydro-1,4-*endo*-*endo*-5-8-dimethanonaphthalene	City Chemical Corp., New York, N.Y.
Chlordane	Technical	1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene	Nutritional Biochemicals Corp., Cleveland, Ohio
Dieldrin	85%	1,2,3,4,10,10-hexachloro-6,7-*epoxy*-1,4,4a,5,6,7,8,8a octahydro-1,4-*endo*-*exo*-5,8-dimethanonaphthalene	Nutritional Biochemicals Corp., Cleveland, Ohio

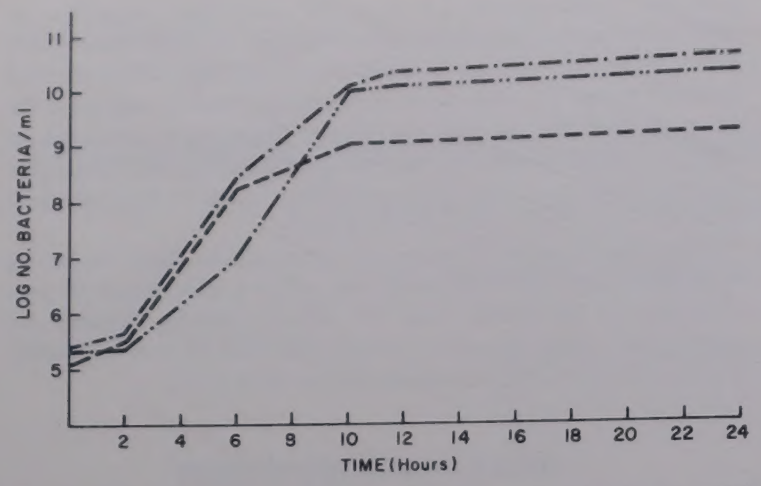

Figure 1. *Growth of* Escherichia coli *in Trypticase Soy Broth (TSB) containing 0, 50 and 100 ppm DDT, Dieldrin or Endrin. Symbols: TSB + 0, 50 or 100 ppm DDT (---); TSB + 0, 50 or 100 ppm Dieldrin (-···-); TSB + 0, 50 or 100 ppm Endrin (-·-).*

Figure 1 shows the growth of *Escherichia coli,* which grew equally well in TSB containing 0 (control), 50, and 100 ppm of either of the three pesticides. Growth curves obtained for *E. coli* in control were similar to curves obtained in TSB containing both concentrations of pesticide. The three pesticides studied caused some differences in growth of the bacteria, but no differences in growth of any of the bacteria were found within a given pesitcide. Similar results were obtained for growth of *Serratia marcescens.*

The three pesticides affected the growth of *Micrococcus luteus, Pseudomonas fluorescens, Streptococcus lactis* and *Lactobacillus lactis* in a similar manner. This effect is illustrated by the growth curves shown in Figure 2 for the growth of *M. luteus* in broth with and without 50 and 100 ppm pesticides. Media con-

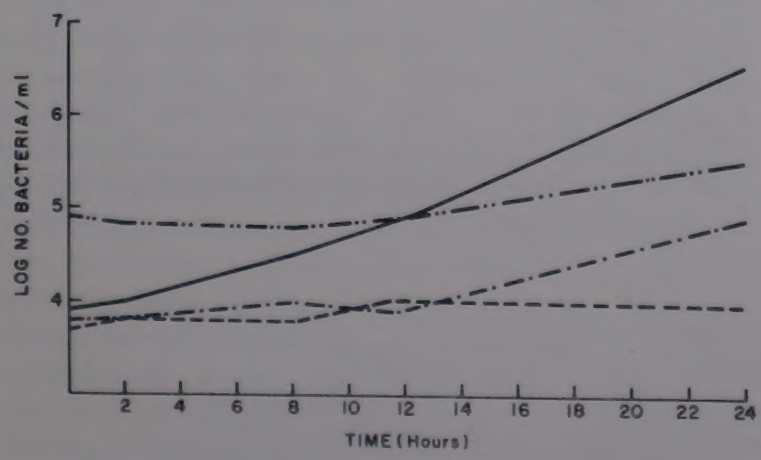

Figure 2. *Growth of* Micrococcus luteus *in Trypticase Soy Broth (TSB) containing 0, 50 and 100 ppm DDT, Dieldrin or Endrin. Symbols: TSB control (——); TSB + 50 or 100 ppm DDT (---); TSB + 50 or 100 ppm Dieldrin (-···-); TSB + 50 or 100 ppm Endrin (-·-·-).*

taining either endrin or dieldrin resulted in greater inhibition during the initial 12 h of incubation than during the final 12 h of incubation. Growth of the four species studied continued to be inhibited after 24 h of incubation in broth containing DDT. No difference in the amount of inhibition was observed between 50 ppm and 100 ppm for any of the three pesticides. All pesticides kept the species in an extended lag phase for a minimum of 12 h before the exponential phase began. A shorter lag phase occured in broth containing dieldrin or endrin than with DDT, which caused the organisms to remain in the lag phase during the entire 24 h incubation period.

The third type of effect obtained is shown in Figure 3. Each pesticide had a different effect on the growth of the

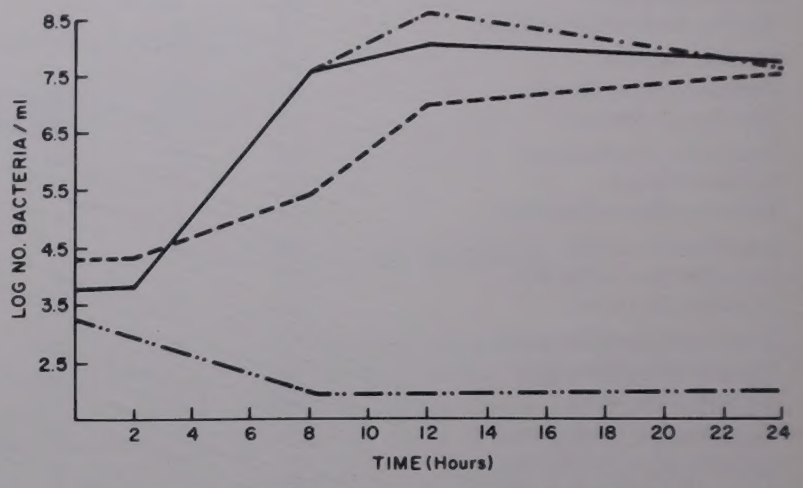

Figure 3. *Growth of* Bacillus cereus *in Trypticase Soy Broth (TSB) containing 0, 50 and 100 ppm DDT, Dieldrin or Endrin. Symbols: TSB control (——); TSB + 50 or 100 ppm DDT (---); TSB + 50 or 100 ppm Dieldrin (-···-); TSB + 50 or 100 ppm Endrin (-·-).*

spore-former, *Bacillus cereus.* No appreciable effect on growth of this species was observed in media containing up to 100 ppm endrin. However, both 50 and 100 ppm DDT caused slight inhibition of growth as shown by an increase in the lag phase. Dieldrin was very inhibitory, causing an initial decrease in count and no increase in count during the 24 h incubation period. The three pesticides had a varied effect on the growth of *Staphylococcus aureus* and *Streptococcus faecium.* Endrin had no effect on their growth, while both species were inhibited by DDT. Dieldrin had a very inhibitory effect on growth of *S. aureus* but very little effect on growth of *S. faecium.*

Effect of chlordane and heptachlor on growth

The effect of 10 ppm chlordane and heptachlor on the growth of 33 bacteria in broth during a 72 h period was determined. The results are given in Table 1.

Growth of 24 bacteria was completely inhibited for up to 72 h in broth containing 10 ppm chlordane or heptachlor. *S. aureus, Bacillus subtilis,* and *Bacillus stearothermophilus* were completely inhibited only by chlordane. Heptachlor had no effect on growth of these three species. All species whose growth was inhibited by both pesticides were gram positive and included members belonging to the genera: *Bacillus, Lactobacillus, Leuconostoc, Microbacterium, Pediococcus, Streptococcus,* and *Sarcina.* Growth of gram negative *Alcaligenes faecalis, E. coli, Proteus vulgaris, Pseudomonas fragi,* and *Pseudomonas fluorescens* was not affected by up to 10 ppm of either pesticide. *Pseudomonas aeruginosa* was affected only by chlordane.

In general, gram negative species showed greater

resistance to inhibition of growth by higher concentrations of pesticides than did gram positive species. Inhibition of growth of gram positive species frequently was observed in broth containing as little as 5 ppm chlordane or heptachlor.

Effects of chlordane and heptachlor on production of acid by lactic acid bacteria

The amount and rate of acid production by 13 lactic acid bacteria and two commercial mixed strain cultures in media containing 5 ppm chlordane or heptachlor are shown in Table 4. Acidity measurements were made at time of inoculation and then every 2 h for 12 h and again at 24 h.

The value shown for 8 h, in general, represents the trend in the production of acid by the species in the presence of the pesticides in skimmilk and Elliker broth. Species, whose ability to produce acid in broth was unaffected by the pesiticdes, began to show an increase in acidity after being incubated for 2 to 4 h; while those species affected by the pesticides showed slight or no increase in acidity after 8 h.

Elliker broth or skimmilk containing either pesticide had little, if any, effect on the ability of *Lactobacillus brevis, Lactobacillus lactis* and *Streptococcus lactis* to produce acid. Amounts and rates of acid production decreased when the remaining 10 lactic acid bacteria and the two mixed strain starter cultures were grown in EB plus lactose containing 5 ppm chlordane or heptachlor when compared with that produced in controls. However, when grown in skimmilk, pesticide concentrations equal to or greater than those used for broth had no effect on acid production by the same organisms. Values did not differ significantly from those obtained for the skimmilk controls.

Variations in the amount and rate of acid produced by the organisms occurred in the broth cultures containing the pesticides. Heptachlor appeared to have a more inhibitory effect on acid production than chlordane for all species. Acid production by both commercial starter

TABLE 4. *Effect of 5 ppm chlordane or heptachlor on ability of selected lactic acid bacteria to produce acid in liquid media*

		Time (hours)														
		0			8			10			12			24		
		Pesticide														
Species	Media	X[c]	C[c]	H[c]	X	C	H	X	C	H	X	C	H	X	C	H
		(% Titratable acidity)														
Streptococcus faecium	M[a]	.23	.23	.23	.35	.33	.32	.43	.42	.43	.53	.53	.52	.83	.83	.80
	E[b]	.28	.29	.29	.50	.28	.29	.59	.27	.27	.60	.27	.24	—	—	—
Streptococcus pyogenes	M	.22	.22	.23	.32	.32	.31	.45	.42	.43	.56	.53	.54	.83	.78	.87
	E	.29	.30	.28	.53	.29	.28	.58	.26	.27	.59	.25	.25	—	—	—
Streptococcus bovis	M	.23	.23	.22	.23	.23	.23	.24	.24	.24	.24	.24	.24	.52	.30	.27
	E	.27	.29	.27	.52	.28	.27	.52	.27	.27	.52	.26	.26	—	—	—
Streptococcus faecalis	M	.20	.22	.22	.35	.35	.33	.41	.41	.40	.45	.44	.43	.70	.69	.69
	E	.22	.22	.22	.41	.21	.22	.49	.23	.23	.54	.27	.23	.62	.56	.23
Streptococcus cremoris	M	.23	.22	.22	.43	.42	.40	.54	.49	.50	.55	.55	.56	.77	.67	.80
	E	.23	.22	.19	.36	.22	.22	.46	.26	.21	.46	.26	.23	.56	.47	.24
Lactobacillus delbrueckii	M	.22	.24	.22	.24	.24	.24	.22	.23	.23	.22	.21	.25	.30	.31	.30
	E	.26	.19	.24	.23	.23	.21	.27	.23	.23	.30	.23	.21	.42	.33	.28
Lactobacillus helveticus	*M*	.22	.22	.22	.28	.28	.28	.33	.30	.32	.34	.34	.30	.65	.55	.60
	E	.22	.22	.23	.40	.23	.41	.46	.26	.21	.50	.30	.22	.58	.51	.24
Lactobacillus acidophilus	M	.25	.24	.24	.26	.25	.25	.29	.27	.28	.30	.30	.31	.56	.50	.53
	E	.19	.22	.21	.34	.24	.25	.46	.26	.26	.53	.28	.27	.69	.39	.32
Lactobacillus bulgaricus	M	.24	.23	.24	.26	.25	.25	.28	.28	.28	.29	.29	.28	.44	.46	.47
	E	.22	.22	.22	.23	.20	.22	.26	.22	.24	.29	.23	.23	.65	.24	.22
Lactobacillus casei	M	.23	.23	.24	.27	.26	.26	.29	.28	.29	.30	.29	.30	.49	.47	.47
	E	.17	.21	.19	.20	.23	.23	.23	.23	.22	.28	.24	.22	.67	.27	.25
Lactobacillus brevis	M	.24	.23	.24	.31	.29	.30	.36	.32	.29	.38	.36	.31	.72	.79	.70
	E	.21	.22	.23	.32	.24	.23	.41	.28	.23	.54	.32	.25	.72	.66	.51
Lactobacillus lactis	M	.25	.25	.24	.26	.25	.27	.29	.28	.28	.27	.28	.26	.74	.65	.69
	E	.21	.21	.22	.31	.26	.23	.38	.31	.24	.41	.36	.29	.70	.62	.46
Streptococcus lactis	M	.23	.22	.23	.27	.25	.26	.27	.26	.27	.30	.30	.30	.40	.39	.38
	E	.23	.23	.24	.33	.24	.23	.39	.27	.24	.48	.28	.30	.67	.66	.62
Mixed strain starter culture[d]	M	.22	.22	.22	.51	.48	.48	.75	.76	.77	.80	.83	.87	.89	.82	.90
	E	.19	.19	.20	.22	.18	.18	.28	.18	.18	.37	.19	.19	.54	.19	.19
Mixed strain starter culture[d]	M	.22	.22	.22	.46	.42	.44	.73	.72	.66	.85	.90	.90	.88	.92	.90
	E	.20	.20	.20	.29	.19	.19	.37	.19	.19	.45	.19	.19	.52	.19	.19

[a]M = Skimmilk with 5 g yeast extract/liter.
[b]E = Elliker Broth with 5% lactose.
[c]X = Control.
[c]C = 5 ppm added chlordane.
[c]H = 5 ppm added heptachlor.
[d] = Commercial mixed strain culture.

cultures was inhibited in broth containing pesticide. However, no differences were observed between the amount of acid produced when the cultures were grown in milk containing chlordane or heptachlor and the amount in the milk controls. *Streptococcus bovis* was the only species studied that produced considerably less acid in milk containing pesticide than was produced in the milk control. This difference was not observed until after 24 h of incubation.

Results of this study were similar to those obtained by Hantke and Bradley (*7*), who found that lactic bacteria produced acid slowly in lactose broth containing dieldrin. However, other researchers (*8, 9, 12*) have reported that organochlorine pesticides have no effect on acid production by lactic acid bacteria. Hantke and Bradley (*7*) concluded that the differences in results were due to acidities not being determined in the other studies during the initial 5 h of incubation when some acid is produced by lactic bacteria.

Effect of chlordane and heptachlor on generation times

Generation times, as determined by periodic plate counts over a 24 h period, were calculated for five species grown in broth and milk media with and without 10 ppm chlordane or heptachlor (Table 5). As previously found in this study, the growth rates of gram negative *E. coli* and *P. vulgaris* were not appreciably affected by the addition of either pesticide to broth. The growth rates of gram positive M. *lacticum* and *S. aureus* were inhibited by both pesiticides when they were grown in broth. However, their growth rate did not appear to be affected when grown in milk containing either pesticide.

The presence of 10 ppm heptachlor in milk seems to cause a decrease in the growth rate for the gram negative bacteria. Generation times obtained for *E. coli, P. vulgaris,* and *P. fluorescens* were greater in milk containing heptachlor than they were in skimmilk controls or in skimmilk plus 10 ppm chlordane.

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TABLE 5. *Generation times of selected bacteria in skimmilk and trypticase soy broth (TSB) with and without heptachlor or chlordane*

Species	Media					
	TSB Control	TSB with 10 ppm Chlordane	TSB with 10 ppm Heptachlor	Skimmilk control	Skimmilk with 10 ppm Chlordane	Skimmilk with 10 ppm Heptachlor
	(Minutes)					
Escherichia coli	25	27	26	28	32	62
Microbacterium lacticum	69	0	0	126	136	130
Proteus vulgaris	37	39	42	40	44	60
Pseudomonas fluorescens	NG	NG	NG	33	30	50
Staphylococcus aureus	30	0	0	35	37	40

NG = No growth.

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Production of Rubratoxin by *Penicillium rubrum* in a Soy Whey-Malt Extract Medium

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ABSTRACT

Sterile soy whey (1.75% dissolved solids) was fortified with malt extract and inoculated with spore suspensions of *Penicillium rubrum* strains P-13, 1062, 2120, 2123, and 3290. Samples were incubated quiescently at 28 C from 1 to 28 days and as shake cultures for 3, 5, and 7 days. Rubratoxin was recovered from culture filtrates by alcohol-acetone extraction and resolved by thin-layer chromatography. Toxin was not produced in shake cultures. Rubratoxins A and B were produced in quiescent soy whey cultures of all *P. rubrum* strains except *P. rubrum* P-13 which produced only rubratoxin B. Toxin production increased as the concentration of malt extract increased from 0.5 to 10% (w/v). Rubratoxin formation also increased with an increase in incubation time from 3 to 17 days but the amount of toxin in cultures declined rapidly thereafter. Yields of rubratoxin A ranged from 0.83 to 31.53 mg/100 ml in cultures of *P. rubrum* 1062 and from 1.89 to 22.70, 0.53 to 25.13, and 2.07 to 31.20 mg/100 ml in *P. rubrum* 2120 2123, and 3290 cultures, respectively. Yields of rubratoxin B ranged from 0.77 to 105.30, 1.03 to 94.83, 2.13 to 91.57, 0.82 to 78.53, and 1.3 to 85.57 mg/100 ml in cultures of *P. rubrum* 13, 1062, 2120, 2123, and 3290, respectively. After maximum production, toxin content in cultures leveled off and then decreased. Amounts of toxin declined more rapidly than did mold growth (as measured by mycelial dry weight). Although malt extract stimulated fungal growth, toxin production was enhanced more than mold growth.

During the past 20 years, several diseases of previously unknown etiology have been attributed to ingestion of foods and feeds contaminated with various *Penicillium* species. In 1953 Sippel et al. (*23*) described a disease of cattle and swine which occurred after they consumed toxic corn. Symptoms of this disease included massive hemorrhage in its acute form or liver hemorrhage in its chronic form. Following extensive mycololgic examination of contaminated corn in 1957, Burnside et al. (*6*) isolated a mold which was later identified as *Penicillium rubrum* Stoll. Wilson and Wilson (*27*) described the isolation of a toxic agent from corn experimentally contaminated with *P. rubrum*. During 1967 and 1968 Moss et al. (*17*) and Hayes and Wilson (*13*) characterized this agent, calling it rubratoxin B. In 1969, Natori et al. (*19*) described the isolation of rubratoxin B from cultures of *Penicillium purpurogenum*. Before this, a second toxic agent, designated as rubratoxin A, had been described by Townsend et al. (*24*).

Although probably noncarcinogenic, rubratoxin B has a synergistic action with aflatoxin (*3*). Furthermore, since *P. rubrum* and *Aspergillus flavus* often grow together under natural conditions, rubratoxin may contribute to the health hazard caused by aflatoxin.

The recent increasing importance of soybean-derived textured vegetable protein as a substitute for beef (especially to meet the needs of developing countries) prompted a study on formation of rubratoxin on soybean substrates. One phase of the study dealt with production of rubratoxin by various strains of *P. rubrum* in soy whey fortified with malt extract. This paper reports the results of the investigation.

MATERIALS AND METHODS

Preparation of inoculum and cultures

P. rubrum P-13 obtained from B. J. Wilson of Nashville, Tennessee and P-1062, 2120, 2123 and 3290 from Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois were maintained as stock cultures on cracked corn moistened with a 1% aqueous sucrose solution (*27*) and were stored at 5 C after the molds had grown. Spore suspensions (5×10^5 conidia/ml) were prepared by washing heavily sporulated cultures of *P. rubrum* with 0.006% Leconal solution (Matheson Scientific, Chicago, Illinois).

Preparation of soy whey-malt extract medium

Soybean kernels (Hark variety) were blended in a Waring Blendor at high speed for 5 min with 15 times their volume of tap water. The resulting mixture (containing soy milk) was filtered, warmed in a water bath maintained at 62 C, and then treated with 1-2 g of anhydrous calcium sulfate, followed by 5 to 10 ml of 6 N hydrochloric acid until protein precipitation was complete. The yellow supernatant fluid (pH 4.5) (soy whey) was recovered by filtration at reduced pressure. Soy whey was fortified with 0.5, 1.0, 2.5, 5.0, and 10% malt extract (Difco Laboratories, Detroit, Michigan) and 100-ml portions of the soy whey-malt extract medium were dispensed into 500-ml Erlenmeyer flasks. All samples were sterilized by autoclaving at 15 psig for 20 min, cooled, and then appropriate samples were inoculated with 1 ml of a spore suspension of each *P. rubrum* strain. Samples were incubated quiescently at 28 C and extracted after 1, 3, 5, 7, 10, 12, 14, 17, 21, and 28 days.

Extraction of rubratoxin

After incubation, mycelia were recovered by filtration through Whatman No. 1 or 42 filter paper at reduced pressure. Rubratoxin was extracted by a modification of the method of Townsend et al. (*24*) as summarized in Fig. 1. The filtrate (100 ml/sample) was concentrated to 20 ml by evaporation at reduced pressure in a Rinco flash evaporator (Rinco Instruments Inc., Chicago, Illinois). After fat removal with petroleum ether (benzine, bp 30-75 C), the aqueous phase of the mixture was extracted twice with an acetone:alcohol (3:1 or 5:1 vol/vol) mixture. The precipitate was removed by filtration, the filtrate (200 ml) concentrated by flash evaporation (45 C) at reduced pressure to 50 ml and then extracted with an ethyl acetate:water (4:1 vol/vol) mixture. The aqueous phase was acidified with 6 N HCl (to pH 1.8) and extracted with diethyl ether (USP). Crude rubratoxin crystals were recovered by a slow evaporation of the ether extract and by freezing the

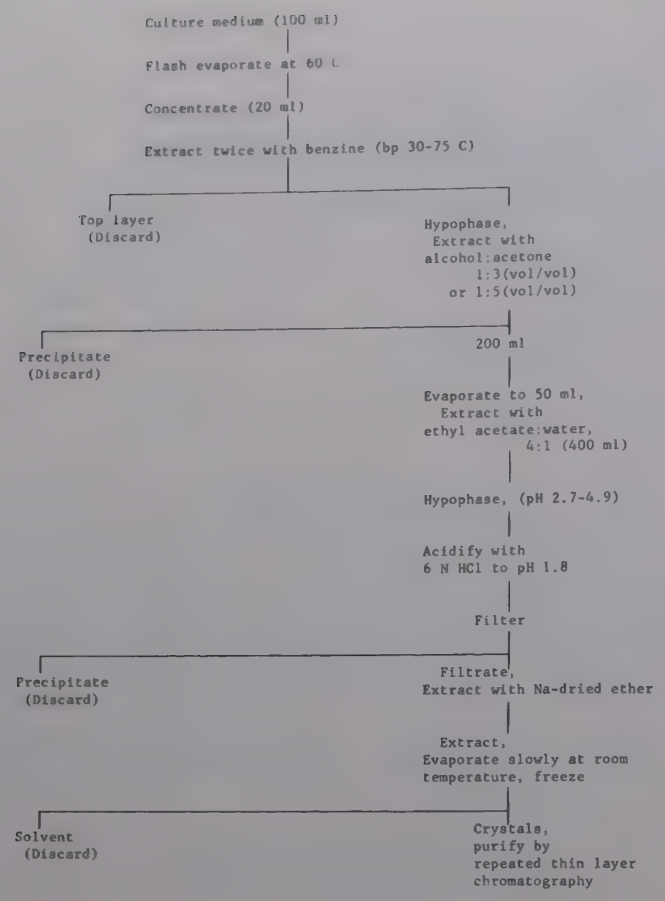

Figure 1. ***Procedure for extraction of rubratoxin A and B from broth cultures of*** **P. rubrum.**

resulting saturated solution at -18 C. Rubratoxins were resolved and purified by thin-layer chromatography.

Resolution, assay, and quantitation of rubratoxin

Crude extracts were dissolved in 100 ml of reagent grade acetone. Standard thin-layer chromatoplates (20 by 20 cm) were coated with a 0.5-mm layer of silica gel HF_{254} (Kensington Corporation, Berkeley, California), activated at 90 C for 2 h, cooled, and were spotted with 40-μl portions of toxin. Chromatoplates were developed 18 cm beyond the origin in an unlined tank saturated with glacial acetic acid:ethyl acetate (15:85 vol/vol). Developed plates were observed under long wave ultraviolet light and rubratoxins identified as blue spots against the fluorescent green background of the plates. Rubratoxins A and B were assayed semiquantitatively by visual comparison with standards obtained from M. O. Moss of the University of Surrey, England. Concentrations of toxins were then determined by reading the ultraviolet absorption profile of samples at 300-200 nm in acetonitrile in a Beckman Acta III Double Beam scanning spectrophotometer (Beckman Instruments, Fullerton, California). Optical density (o.d.) determinations were made for rubratoxin A at 252 nm and at 251 nm for rubratoxin B using molar extinction coefficients recommended by Moss et al. (*18*). Toxin yields were calculated from a standard curve, taking into account the dilutions involved.

RESULTS

Strain P-13

This strain of *P. rubrum* produced only rubratoxin B in amounts which ranged from 0.77 to 104 mg/100 ml medium (Fig. 2). The mycelium developed rapidly (after 24 h of incubation) and covered the entire surface of the medium by the 7th day of incubation. A marked drop in pH occurred in all cultures (Fig. 2). The pH increased rather rapidly after 12 to 14 days of incubation, the rate of increase being inversely proportional to the concentration of malt extract in the medium.

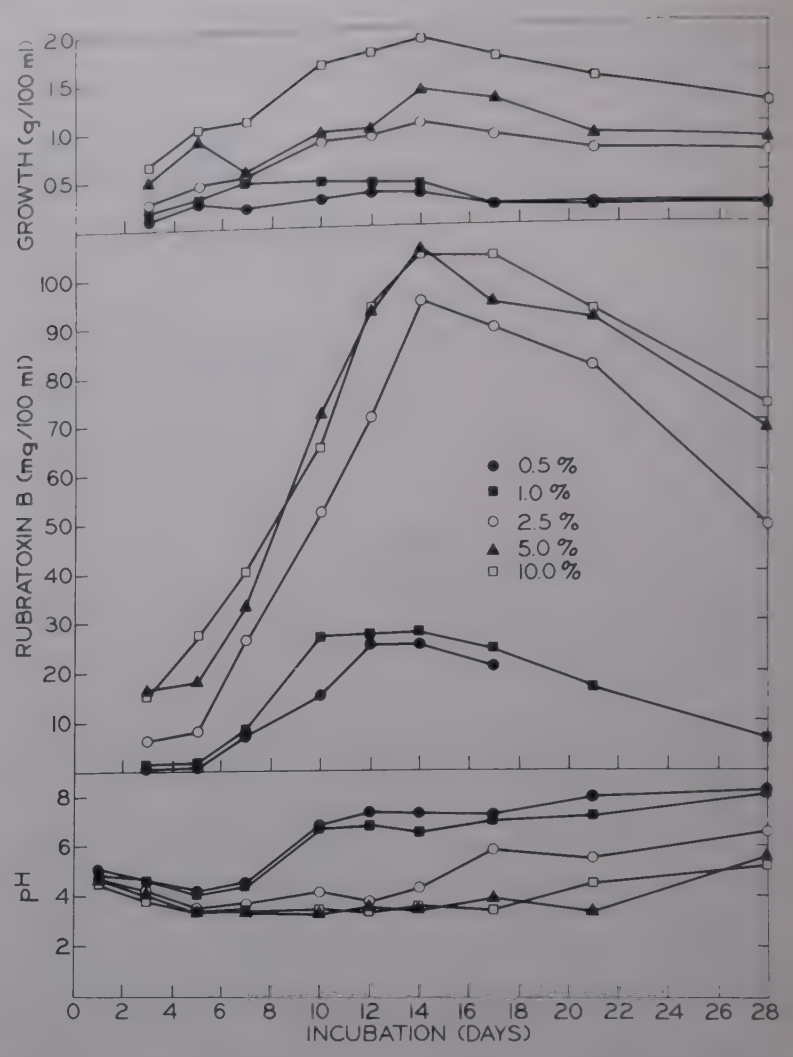

Figure 2. ***Growth, changes in pH, and rubratoxin synthesis by*** **Penicillium rubrum** ***13 at 28 C in soy whey with various concentrations of added malt extract.***

Strain P-1062

Both rubratoxins A and B were produced by this strain of *P. rubrum* although rubratoxin B was the more abundant compound (Fig. 3). Amounts of rubratoxin B recovered from mold cultures varied from 0.83 to 30.53 mg/100 ml (Fig. 3). The total amount of rubratoxin recovered from cultures of this *P. rubrum* strain increased with the period of incubation and maximum yields occurred at 14 and 21 days of incubation, (5% malt extract-fortified cultures, Fig. 3). There was a gradual decrease in pH at 5 to 14 days of incubation, the pH rising rapidly thereafter. Generally, growth of *P. rubrum* 1062 showed a monophasic variation with time of incubation [i.e. increased with incubation until a maximum amount was attained, then declined(*1*)].

Strain P-2120

This strain produced both rubratoxins A and B (Fig. 4). Rubratoxin B was produced at 3 to 28 days in cultures fortified with 2.5, 5.0 and 10% malt extract, and at 3 to 17 or 21 days in cultures fortified with 0.5 and 1.0% malt extract, respectively. Rubratoxin A was produced sporadically for only brief periods (7 to 14 or 7 to 28 days). Growth of the mold was rapid and showed a monophasic variation with period of incubation (Fig. 4).

Strain P-2123

Both rubratoxins A and B were produced by *P.*

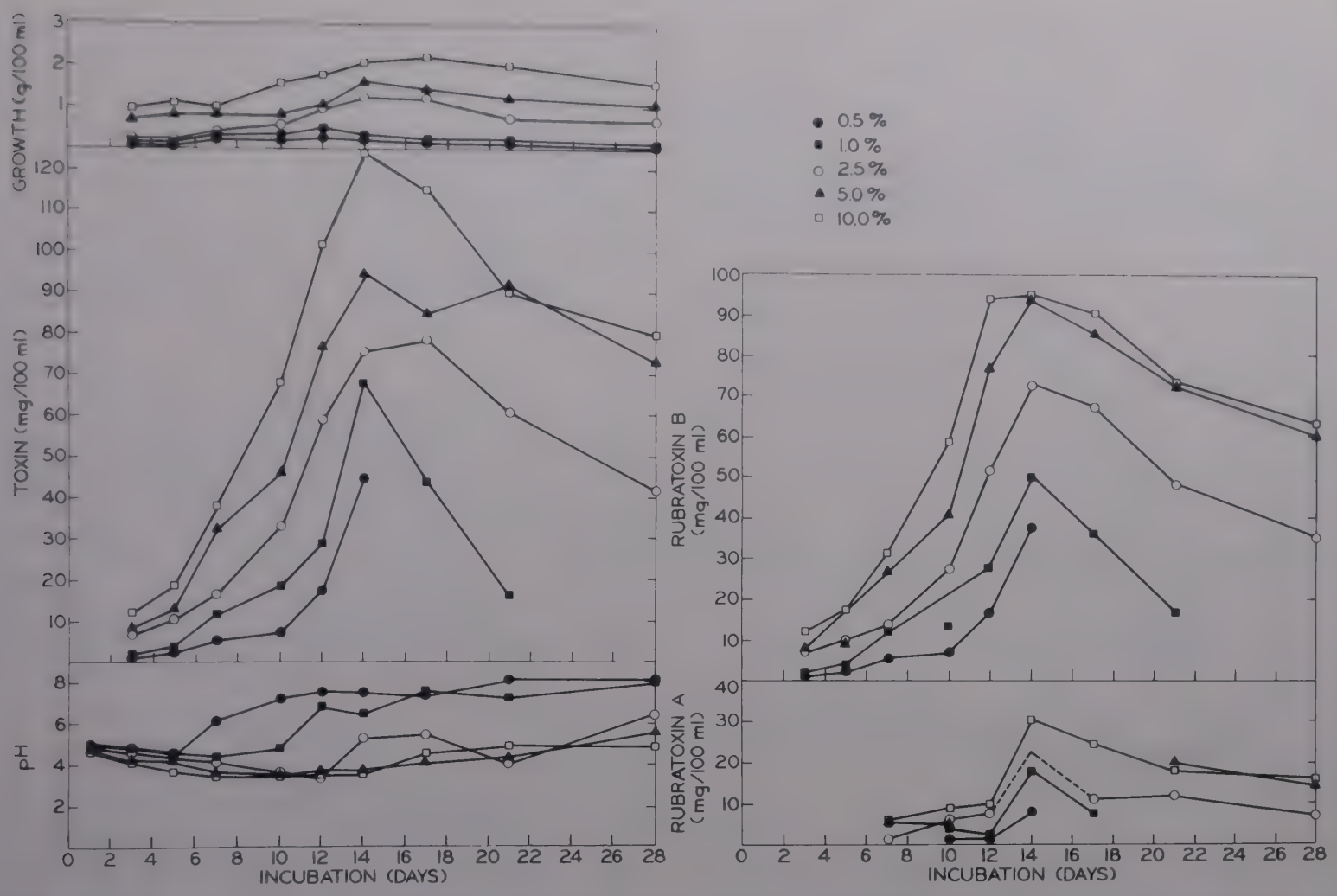

Figure 3. *Growth, changes in pH, and rubratoxin synthesis by* Penicillium rubrum *1062 at 28 C in soy whey with various concentrations of added malt extract.*

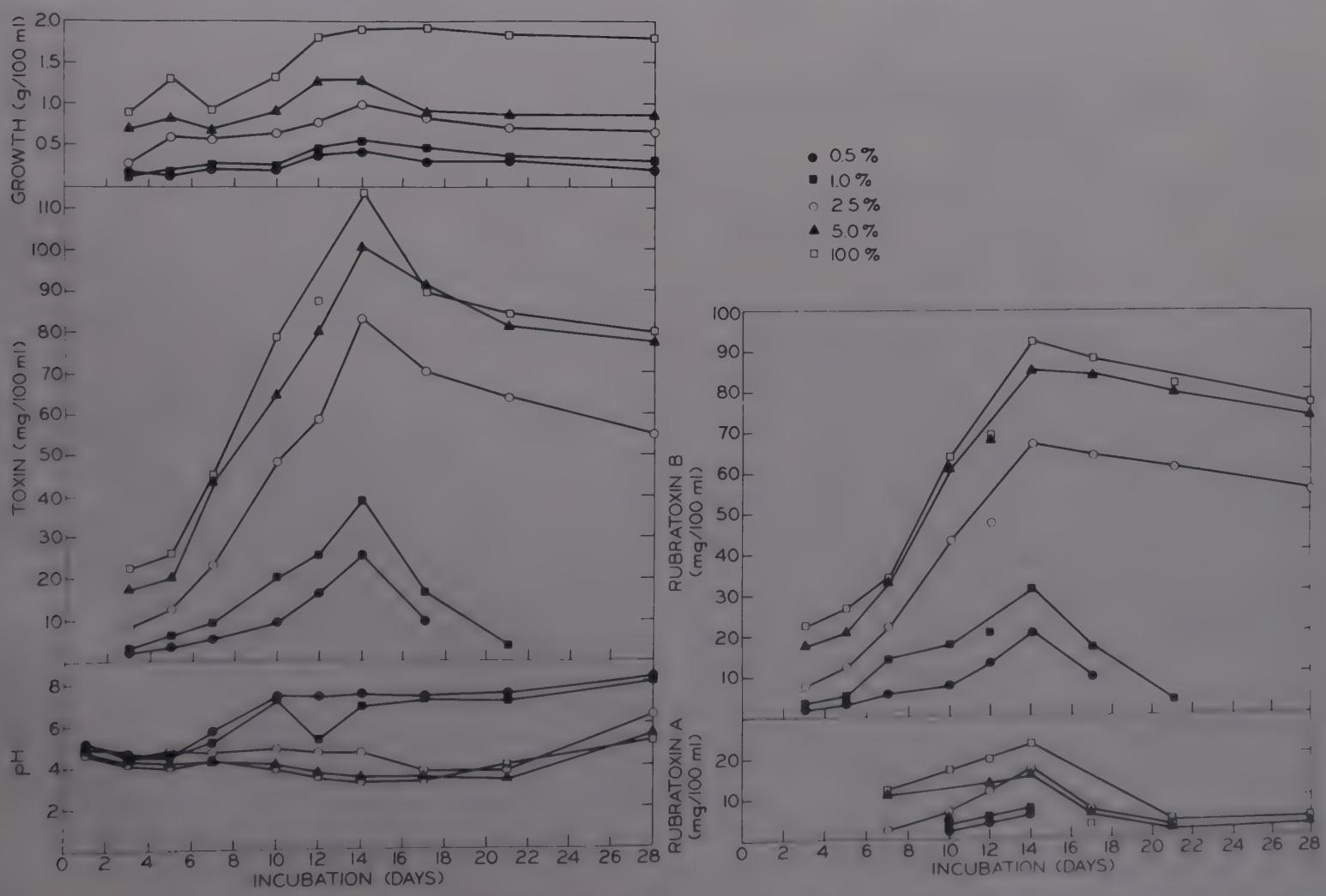

Figure 4. *Growth, changes in pH, and rubratoxin synthesis by* Penicillium rubrum *2120 at 28 C in soy whey with various concentrations of added malt extract.*

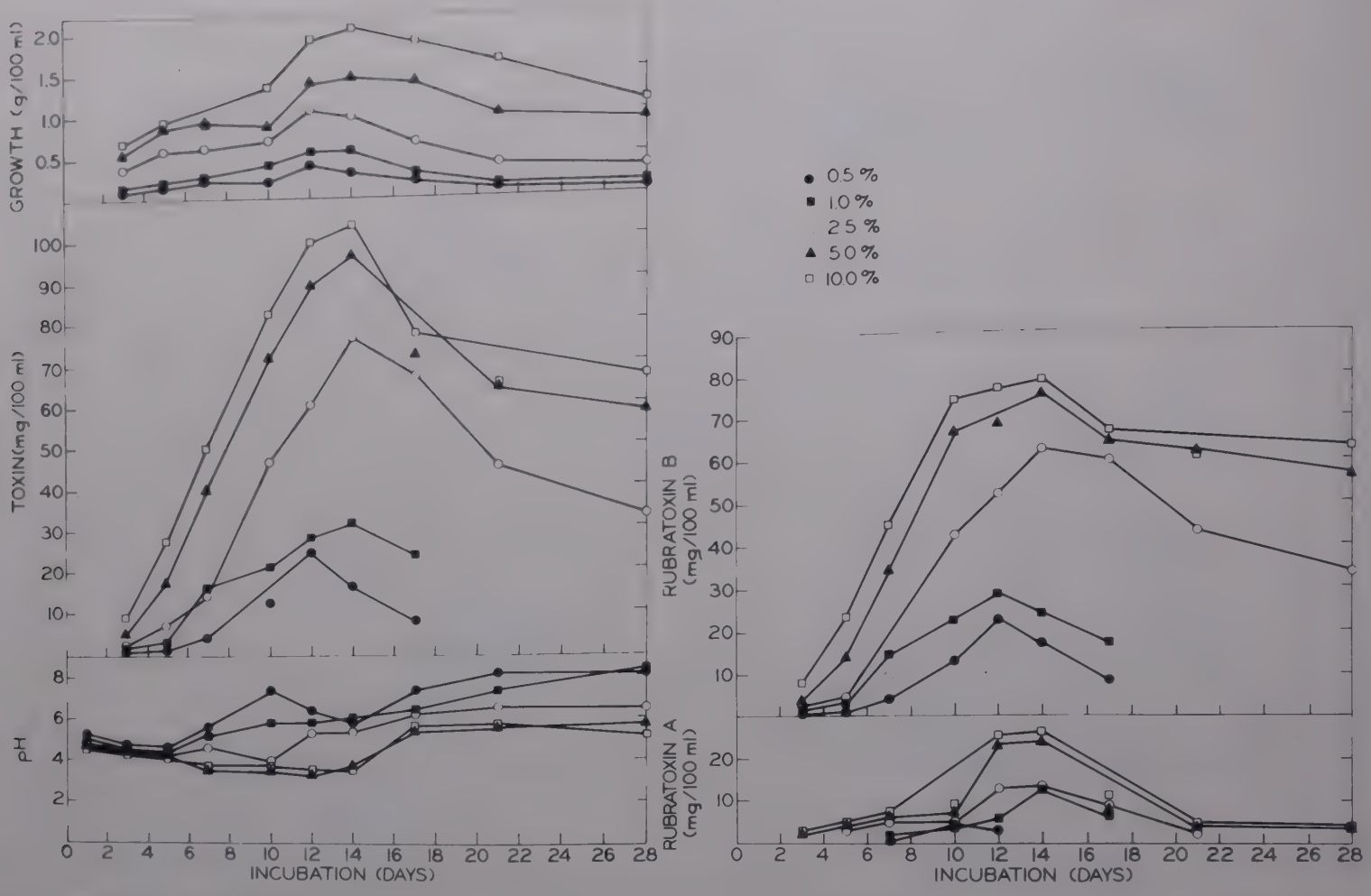

Figure 5. *Growth, changes in pH, and rubratoxin synthesis by* Penicillium rubrum *2123 at 28 C in soy whey with various concentrations of added malt extract.*

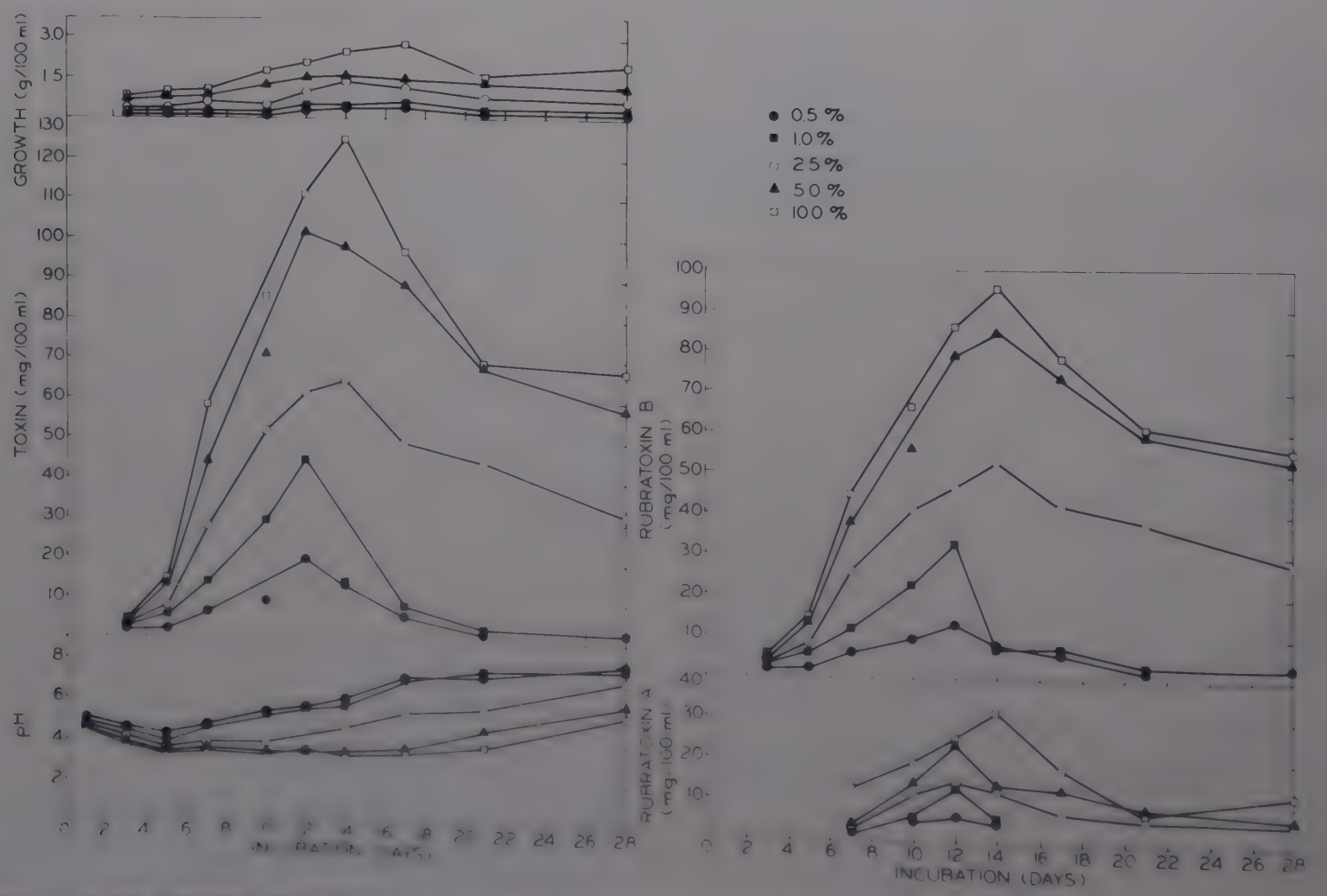

Figure 6. *Growth, changes in pH, and rubratoxin synthesis by* Penicillium rubrum *3290 at 28 C in soy whey with various concentrations of added malt extract.*

rubrum P-2123. Growth of the mold was not evident after the first day but was rapid thereafter. This growth was accompanied by a rapid decrease in pH, which showed a biphasic variation with period of incubation (i.e. declining, leveling off, and rising at two different times, Fig. 5). Yields of both rubratoxins A and B increased with period of incubation, leveled off after maximum toxin production, and then decreased. Toxin levels, however, showed a biphasic variation with growth (Fig. 5).

Strain P-3290

This strain of *P. rubrum* which produced both rubratoxins A and B (Fig. 6), also produced more toxin than other strains (Fig. 6). Growth and toxin formation showed a monophasic variation with time of incubation (i.e. increasing to a maximum, leveling off, and then decreasing). The pH profile showed an inverse relationship to time of incubation; the rate of pH increase also being inversely proportional to malt extract concentration in the medium.

DISCUSSION

The time-course of rubratoxin accumulation in vitro has been described as monophasic by Wilson and Wilson (*27*) and Hayes and Wilson (*13*). Recently, however, Moss and Hill (*16*) showed accumulation of rubratoxin to be biphasic. Our results show that the pattern of rubratoxin synthesis by *P. rubrum* is strain-dependent.

Preliminary experiments in our laboratory showed that soy whey alone did not support toxin production. All strains of *P. rubrum* used in this study produced mostly rubratoxin B. Although the maximum rate of toxin accumulation occurred during the period of maximum growth (as measured by mycelial dry weight), an increase in mold growth was not always accompanied by an increase in toxin production (Fig. 2, 6), (Emeh and Marth, unpublished data). The period of toxin synthesis occurs rather early in the growth cycle of the fungus and this suggests a possible relationship between the reactions essential for growth and those involved in rubratoxin formation.

The overall effect of malt extract on toxin formation is shown in Fig. 7 and 8. Generally, an increase in malt extract concentration stimulated rubratoxin formation. However, optimal synthetic efficiency (as measured in mg of toxin/g dry weight of mycelium) was favored by addition of 2.5% malt extract to the culture medium. Variation existed among the strains not only in the amount of either rubratoxin A or B produced but also in the amount of either toxin produced by each *P. rubrum* strain in different experiments [(Emeh and Marth, unpublished data; and Moss and Hill (*16*)]. It is quite possible that parent cultures of the isolates used in this study could have behaved differently under similar experimental conditions, as has been observed in aflatoxin formation by Davis et al. (*9*).

Growth was not recorded until after the first day of incubation because of the assay procedures adopted in this study. After this time, however, growth was rapid and increased with an increase in malt extract concentration (Fig. 2-6), reaching a maximum at 12 to 14 days and declining thereafter. The variation in the rate of decline (g dry weight/time) was a reflection of malt extract concentration (*12, 22*). A high concentration of malt favored a less gradual decline of growth and toxin production (Fig. 2-6).

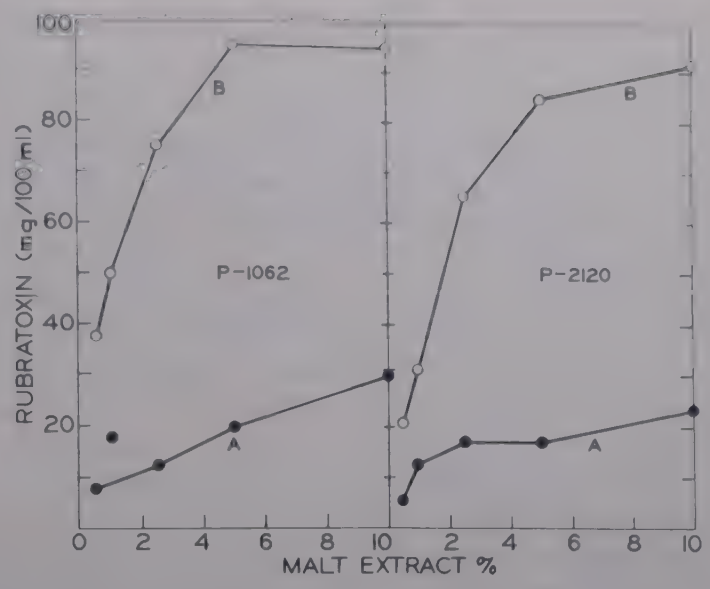

Figure 7. *Overall effect of malt extract on the maximum amounts of rubratoxins A and B recovered from cultures of* Penicillium rubrum *P-1062 and P-2120.*

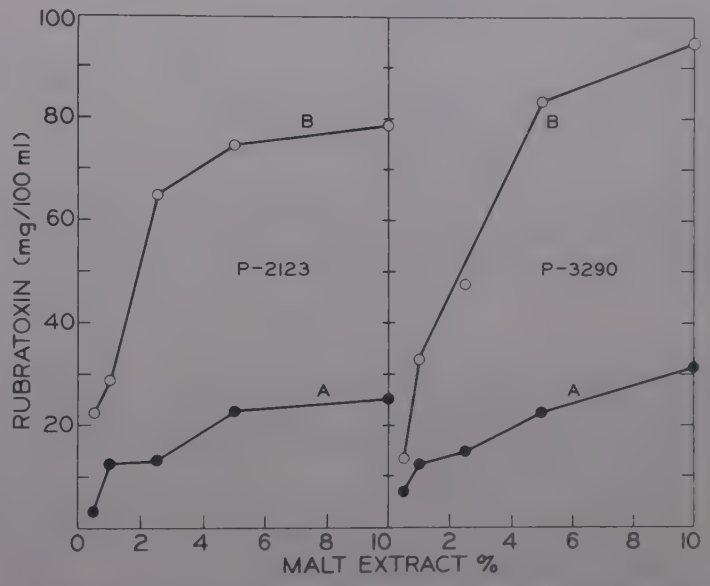

Figure 8. *Overall effect of malt extract on the maximum amounts of rubratoxins A and B recovered from cultures of* Penicillium rubrum *P-2123 and P-3290.*

It is assumed that growth declined as the nutrients in the medium were depleted and that the mold began to degrade the rubratoxin using degradation products for its metabolism. Microbial metabolism of mycotoxins especially aflatoxin has been described by Ashworth et al. (*2*), Ciegler et al. (*8*), and Jarvis (*15*). Several β-lactones are known to undergo nucleophilic attack leading to ring opening in the presence of OH^- ions (*4, 10*). Whether or not this occurs with rubratoxin has not been determined.

A low pH (Fig. 2-6) (usually below 5.0 but often below 4.0) accompanied optimal toxin synthesis, the amount of toxin decreasing rather sharply as the pH increased

above 7.5 (*5, 11*). The high final pH generated, especially in older cultures with low malt extract supplementation (0.5 and 1.0%), was accompanied by disappearance of toxin or decrease in toxin yields. This may be caused by release of ammonia produced by oxidative deamination of the protein in the medium.

Deterioration of soybeans by molds constitutes a serious economic problem because of the increasing importance of soy-based textured vegetable protein in the preparation of hamburgers, meat extenders, breakfast and other foods (*7, 20*). It is also likely that improved technology will facilitate production of convenience foods, feeds, and feed supplements from soy whey protein (*14, 25*).

The acute shortage of food in the developing world most notably and most recently the famine in the Sahel region of West Africa and northern Ethiopia has made development of safe, cheap, high protein food top priority. Soybean products will help to alleviate this situation. We have studied production of rubratoxin on various soybean substrates. Results of that investigation will be reported in a future communication.

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A Strain of *Pseudomonas aeruginosa* Resistant to a Quaternary Ammonium Compound

I. Physiological Properties

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ABSTRACT

Pseudomonas aeruginosa cells were selected for their ability to grow in the presence of 770 ppm n-alkyl (50% C12, 30% C14, 17% C16, 3% C18) dimethyl dichlorobenzyl ammonium chloride (QAC). These cells retained resistance to the germicide throughout tri-weekly transfers for 7 months in tryptone glucose yeast extract broth containing no QAC. Comparisons of resistant and sensitive cells were made in an attempt to define the mechanism of resistance and to provide some information as to the mode of action of QAC. Broth cultures of the resistant strain displayed a distinct fruity odor. Gas chromatographic analysis showed that the QAC-resistant cells, unlike the sensitive, produced large quantities of ethyl acetate and ethyl valerate. Two bands of esterase activity were demonstrated in sensitive cell extracts by gel electrophoresis, while only one band was detected in the resistant cell extracts when alpha napthyl acetate was used as the substrate. Biochemical tests disclosed numerous differences between the two cell types, many of which appeared to be interrelated. The most significant differences were losses in the ability of resistant cells to synthesize extracellular lipase and protease enzymes. Many other biochemical tests on resistant cells were negative or became positive only after prolonged incubation. Resistant cells were also more resistant to osmotic disruption. Permeability studies indicated a reduced rate of glucose uptake by resistant cells. Furthermore, growth curve studies indicated a slower rate of growth by resistant cells and a 15-min longer generation time.

Quaternary ammonium compounds (QAC) have gained widespread use in the past decade, especially in pharmaceuticals and as skin disinfectants. Lawrence (*18*) and Lawrence and Block (*19*) have published review articles on the research conducted on QAC. The development of resistance by bacteria to those antimicrobial compounds has been well documented; for example, Dyar and Ordal (*12*) reported on a QAC-resistant strain of *Micrococcus aureus* (*Staphylococcus aureus*). Chaplin (*9*) was unable to confirm this in *S. aureus*, but did obtain resistance in *Escherichia coli* and *Serratia marcescens*. Recently Maxcy et al. (*24*) reported some of the characteristics of a QAC-resistant culture of *E. coli*. MacGregor and Elliker (*21*), Soprey and Maxcy (*30*), and Adair et al. (*1*) have studied the resistance of *Pseudomonas aeruginosa* to QAC and Anderes et al. (*4*) have provided an extensive lipid analysis of the sensitive and resistant strains of this organism. In spite of the popularity of this topic, the mechanism of action of QAC is not clearly defined nor has the resistance phenomenon been fully elucidated. The intent of the present investigation was to compare sensitive and resistant cells to better understand the effects of QAC and the changes occurring in cells acquiring resistance.

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MATERIALS AND METHODS

Strains of bacteria used

P. aeruginosa QRM-S came from the culture collection maintained in the Department of Microbiology at Oregon State University. The resistant strain QRM-R was developed by subculture of the sensitive strain in broth medium (see below) containing sub-lethal germicide concentrations with incubation for up to 7 days at 32 C. It was maintained in broth containing 514 ppm QAC. A third strain, QRM-RN, resulted from a minimum 7 months transfer of the resistant strain in the absence of germicide.

Germicide

The germicide used in this study was the QAC n-alkyl (50% C12, 30% C14, 17% C16, 3% C18) dimethyl dichlorobenzyl ammonium chloride (Klenzade Corporation, Division of Economics Laboratory, Minneapolis, Minnesota).

Media

Routine propagation of the sensitive strain was carried out in tryptone-glucose-yeast extract broth (TGY) containing 0.5% tryptone, 0.25% glucose and 0.1% yeast extract. The resistant strain was propagated in TGY broth containing 514 ppm germicide. When a solid medium was desired, 1.5% agar was incorporated. QAC was added after the medium had been sterilized by autoclaving.

Death curves

P. aeruginosa QRM-S and QRM-R cells were grown to stationary phase in TGY broth and TGY broth plus 514 ppm QAC, respectively. Both cell types were harvested by centrifugation and resuspended in 50 ml of sterile physiological saline. An initial plate count was made on the cell suspensions using TGY agar and plates were incubated at 32 C for 48 h. After removing an aliquot for plating, 50 ml of a 514-ppm QAC solution was added to each suspension to give a final germicide concentration of 257 ppm. The number of viable cells was determined by the plate count method using 1.0% tryptone in the dilution blanks to inactivate the QAC. Controls consisted of each washed cell suspension in physiological saline containing no QAC.

Generation time

P. aeruginosa strains QRM-R, QRM-RN, and QRM-S were grown to late log phase and diluted 1:100. One milliliter of the dilution served as the inoculum for one liter of TGY broth. The initial cell count was determined using TGY agar and the flasks were incubated at 32 C on a rotary shaker.

Permeability studies

Sensitive cells were grown for 14 h in TGY broth and resistant cells were grown for 18 h in TGY broth containing 514 ppm QAC. A 1% inoculum was then made into TGY broth and the cultures were

incubated on a rotary shaker at 32 C, the sensitive cells for 6 h and the resistant cells for 8 h. Cells were harvested and washed twice with pH 7.0 sodium phosphate buffer (0.05 M) to which 50 μg of chloramphenicol/ml had been added. The cells were resuspended in flasks containing 20 ml of buffer. These flasks, and an additional control flask containing 3 mg of glucose/ml, were placed in a 37-C water bath. Once the temperature had equilibrated, 6 ml of cells were mixed with 3 ml of glucose solution. At 0, 15, 30, and 60 min a 0.4-ml aliquot was removed and diluted to 4 ml with buffer. Cells were then centrifuged and the supernatant fluid was retained for glucose assay, according to the procedure of Dische (*11*). The viable count was determined on the original cell suspension and the glucose content was expressed as μg per tube per 4.0×10^{10} cells.

Enzyme assays

The level of proteinase activity in cell-free extracts of *P. aeruginosa* QRM-S and QRM-R was measured using Azocoll (Calbiochem) as the substrate. The A_{580} was determined on the filtrates and the values then used as a measure of the activity. The method of Lowry et al. (*20*) was used to determine the protein concentration in the preparation. The data were expressed as milligrams of protein per milliliter.

The ability to synthesize extracellular lipase was determined by inoculation of bacterial masses on spirit blue agar (*32*). Plates were incubated at 32 C and observed after 18 and 24 h and periodically during 4 weeks at 10 C.

Total lipid

To observe cells for lipid inclusions, smears of cell suspensions were fixed on glass slides and stained with Sudan Black B according to the procedure of Burdon (*8*).

Sensitive cells were grown for 24 h in TGY broth on a rotary shaker at 32 C. The broth medium for resistant cells contained 257 ppm QAC and a 30-h incubation period was used. Cells were harvested from the broth, washed three times in cold sterile physiological saline solution and then lyophilized. The dried cells were weighed into 15-ml Konte flasks, 10 times the cell weight of chloroform:methanol (2:1 vol/vol) were added and the mixture refluxed for 30 min in a 75-C water bath. The cellular material was precipitated by centrifugation at 14,000 rpm and the solvent was decanted. The solvent-extracted cells were dried and weighed as was the lipid-laden solvent. The lipid content was expressed as percent of dry weight.

The effect of possible lipid precursors was also investigated. Cultures were grown several times in media containing one of the following: 0.2% acetic acid, 0.2% sodium acetate, 0.2% sodium citrate, 0.1% sodium pyruvate, 0.1% glycerol, or 100 to 1,000 ppm of acetone. The resistance of these cultures was then determined in TGY broth containing both QAC and the appropriate precursor. In addition, both the sensitive and resistant strains were maintained for 15 weeks in media containing 3.0% glycerol in an attempt to enrich the cells.

Treatment with lipase

Washed cells were incubated for 1 h in the presence of 1.1 mg lipase/ml. These cells were used to inoculate a series of TGY broth tubes containing progressively greater amounts of QAC. During incubation for 7 days at 32 C, tubes were observed for pellicle formation and growth was spotted on TGY agar.

Gas chromatography

Late log phase cells of *P. aeruginosa* QRM-S were inoculated at the rate of 1% into 20 ml of TGY broth in large screw-capped test tubes and incubated on a reciprocal shaker for 24 h at 32 C. Resistant cells were inoculated into both TGY broth and TGY broth containing 514 ppm QAC and incubated for 48 h.

The head space was analyzed for volatile compounds by the oncolumn trapping, gas-liquid chromatographic procedure of Morgan and Day (*25*). Vials containing 8 ml of culture were equilibrated to 50 C. Nitrogen was bubbled through the samples for 5 min to carry the entrained volatiles into the column trap. A 0.318 by 366 cm column packed with 60 to 80 mesh Celite 545 coated with 20% 1,2,3,-tris-(2-cyanoethyoxy) propane was used. The flow rate of nitrogen gas was 24-30 cc/min at a column temperature of 70 C. The gas chromatographic peaks were identified on the basis of retention time.

Gel electrophoresis

Late log phase cells of the sensitive and resistant strains were harvested from TGY broth by centrifugation and resuspended as heavy slurries in sterile distilled water. About 2.5 ml of the slurry were placed in a homogenator container and 7.5 g of glass beads were added. The container was then placed in a Braun Model MSK mechanical cell homogenizer (Bronwill 2876) for 1 min. The slurry was then centrifuged for 10 min at 2,000 rpm and the supernatant fluid was recovered and placed in an ice bath.

Gel electrophoresis was done by the procedure of Davis (*10*). Two hundred microliters of various proportions of large pore gel-sample mixtures were layered on top of the gel columns. Following electrophoresis, protein bands were stained with Amido Schwarz. The esterase bands were determined by the methods of Markert and Hunter (*23*) and Hunter and Maynard (*16*) using *a*-napthyl acetate and staining with Fast Blue RR.

Biochemical methods

Several biochemical comparisons were made employing the procedures used in the taxonomic study by Stanier et al. (*31*) and the tests given in *Bergey's Manual of Determinative Bacteriology* (*7*) and outlined in several other sources (*3, 6, 13*).

RESULTS

The initial phase of this study required the development of a QAC resistant bacterial strain. The resistant strain grew consistently in TGY broth containing 770 ppm QAC and occasionally in the broth with 1015 ppm. Faster growth rate and convenience led to the use of 514 ppm in the transfer broth. The resistance was established by comparing the survival curves (Fig. 1) of both sensitive and resistant strains in

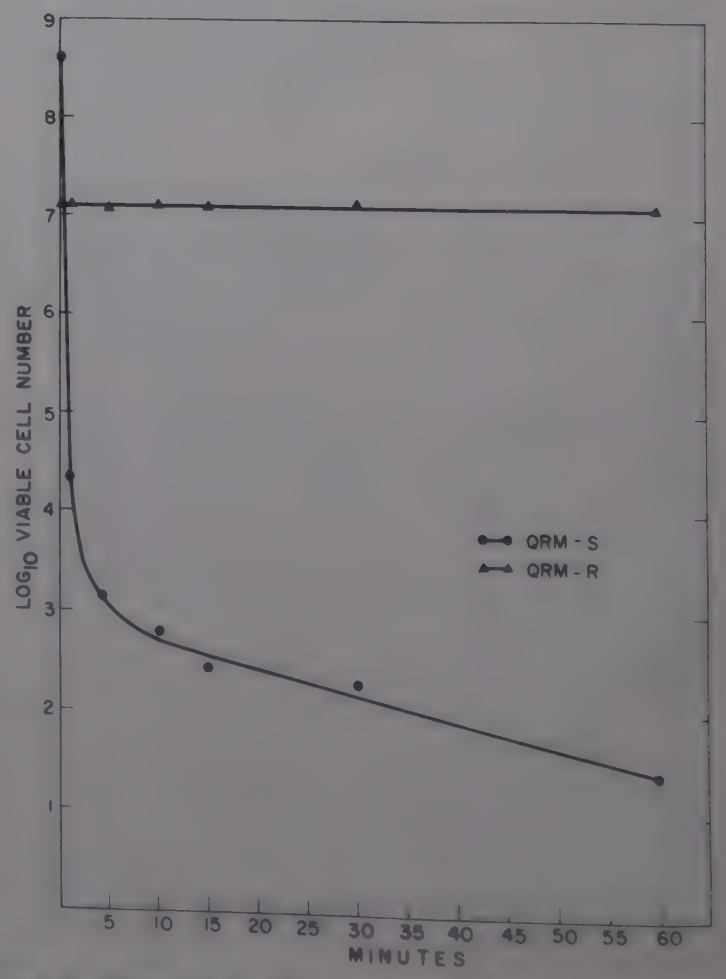

Figure 1. *Effect of exposure of QRM-S and QRM-R cells of* Pseudomonas aeruginosa *to 257 ppm QAC for the times indicated.*

257 ppm of QAC. The difference was quite pronounced; little germicidal activity was evident against QRM-R in 60 min while > 99.9% of the sensitive population was not recovered following 1 min of exposure.

Characteristic growth of cultures

It was noted during the transfer and maintenance of cultures that turbidity and pellicle formation was much more rapid in sensitive cultures. Figure 2 furnishes a

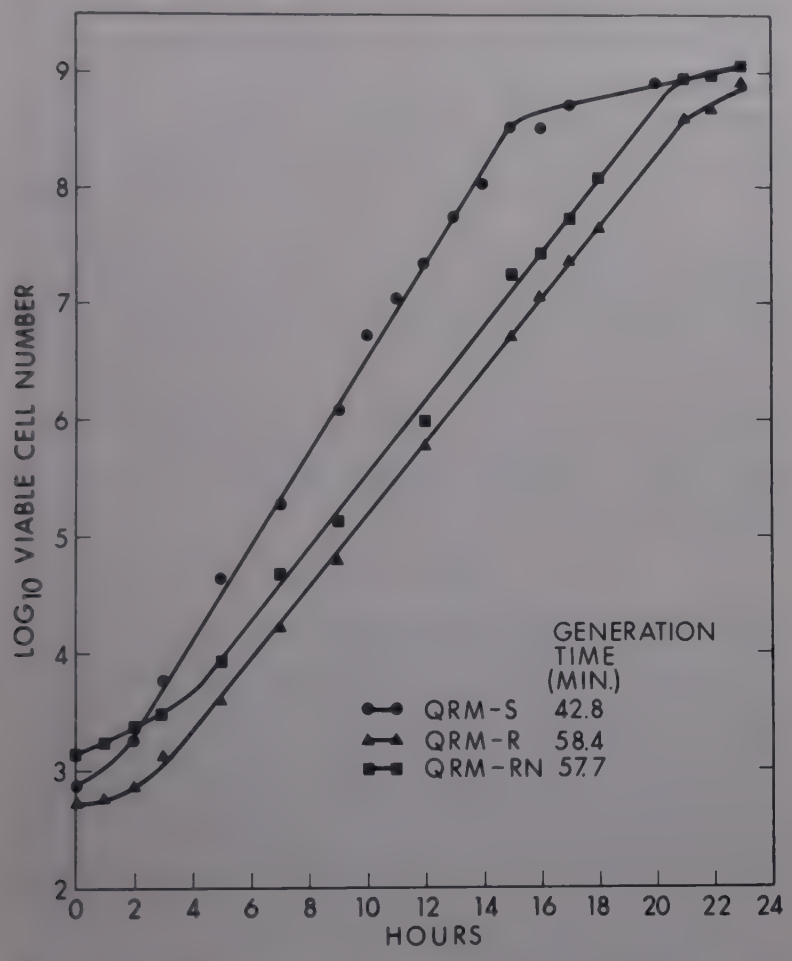

Figure 2. *Growth curves of* Pseudomonas aeruginosa *strains QRM-S, QRM-R and QRM-RN in TGY broth incubated at 32 C on a rotary shaker.*

comparison of the growth curves of QRMS-S, QRM-R, and QRM-RN cultures. The sensitive cells had a generation time of 42.8 min in log phase while the generation time of resistant cells was 58.4 min, 25% longer than that of sensitive cells. The sub-culturing of the resistant strain in the absence of QAC for 7 months (QRM-RN) did not alter this generation time.

Cellular permeability

The rate of glucose uptake for resistant cells was much less than that for sensitive cells. Figure 3 shows that the amount of glucose taken up by sensitive cells in 5 min exceeded the resistant cell uptake over a 60-min period.

Attempts to alter the cell surface or cell membrane characteristics with toluene or dimethyl sulfoxide (DMSO) did not affect the QAC susceptibility of either the sensitive or resistant strain.

Proteinase and lipase activities

The proteinase activity of cell sonicates is shown in Fig. 4. The activity of this enzyme is shown to be much

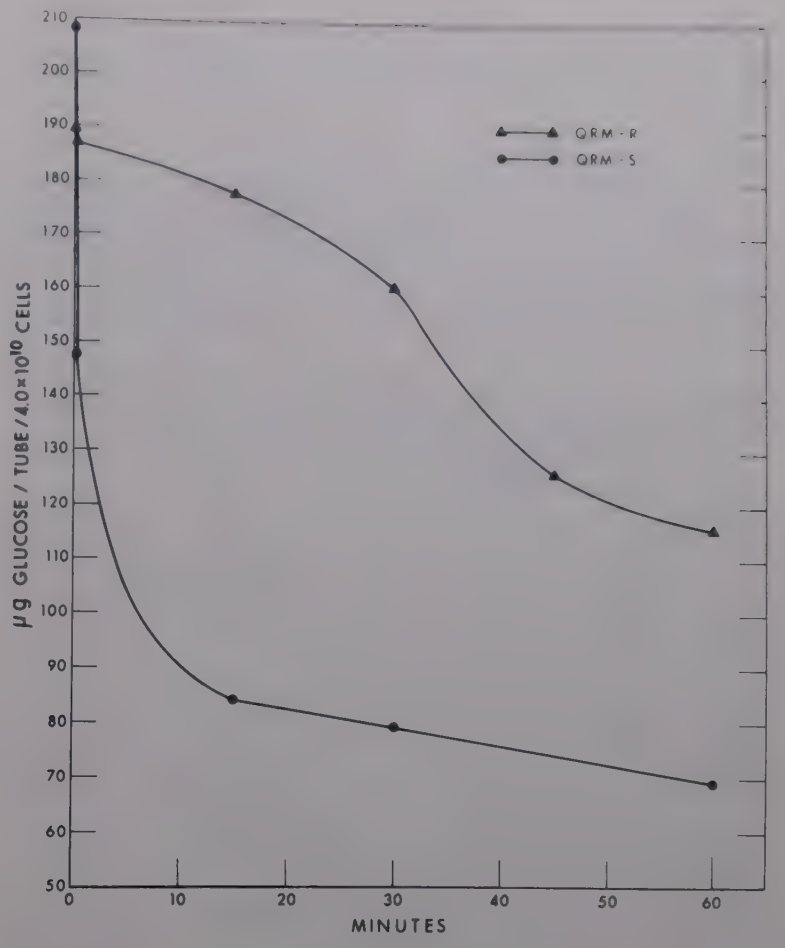

Figure 3. *Glucose uptake by resting cells of QAC-sensitive and QAC-resistant strains of* Pseudomonas aeruginosa.

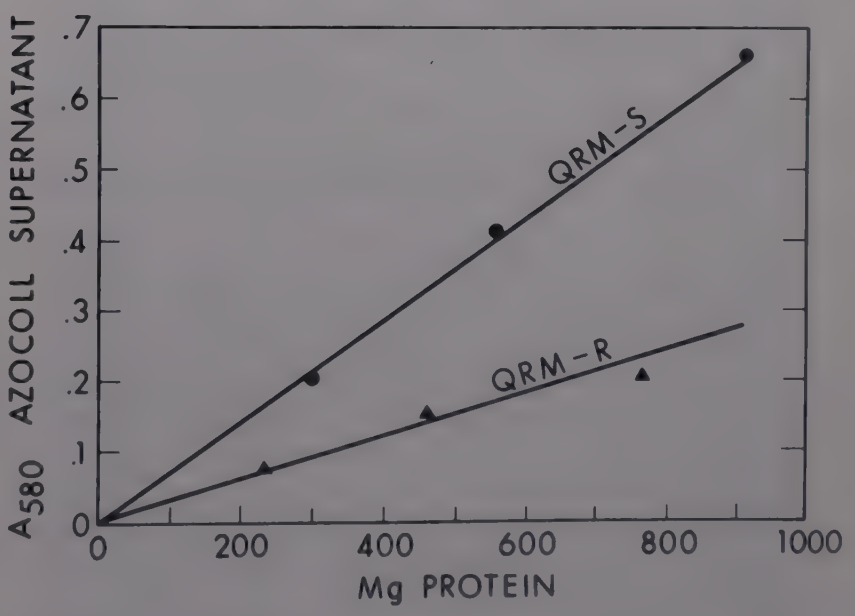

Figure 4. *Comparison of the proteolytic activity of sonicates of sensitive and resistant strains of* Pseudomonas aeruginosa.

less in the resistant cells. Similarly, extracellular lipase activity, as measured by Spirit Blue agar, was produced only by the sensitive cells.

Lipid studies

Sudan Black staining revealed a preponderance of sudanophilic material in both the QRM-R and QRM-RN strains of *P. aeruginosa.* The sensitive parent organism did not display such an accumulation.

Quantitation revealed that QRM-S contained 8.4% and QRM-R 12.5% lipid. Treatment of sensitive and resistant cells with lipase did not alter the degree of

resistance to QAC. Attempts to enrich the cells by feeding lipid precursors to increase resistance were not successful.

Gas chromatography

Detection of a fruity odor produced by the resistant cells and the apparent absence of the odor in sensitive cultures led to GLC analysis of the head space of both cultures. From the results (Table 1), it appears that the sensitive and resistant strains used the acetaldehyde and the propanal present in TGY broth and that both produced ethanol. It also seems that only the sensitive cells produced 2-butanol. Third, the fruity ethyl esters, ethyl acetate and ethyl valerate, were produced extensively by resistant cells, but only traces were elaborated by sensitive cells.

TABLE 1. *Gas chromatographic head space analysis of TGY broth cultures of* Pseudomonas aeruginosa *QRM-S and QRM-R*

T_R (cm)	cm^2 Peak area			Peak identity
	TGY Broth	QRM-S	QRM-R	
4.5	—	2.7	1.2	?
5.2	24.0	0.6	3.5	acetaldehyde
9.2	10.5	—	—	propanaldehyde
12.8	17.6	2.3	48.9	ethyl acetate
13.8	356.0[a]	—	—	QAC
14.8	—	—	60.5	?
15.3	—	53.5	24.7	ethanol
16.8	53.6	—	10.8	?
18.2	—	—	—	ethyl propionate
19.6	6.5	—	—	
23.3	—	—	—	ethyl butyrate
24.0	—	4.9	—	2-butanol
26.5	—	—	6.7	?
29.6	—	1.9	82.8	ethyl valerate
30.8	—	—	16.4	?
32.4	—	5.25	—	?

[a]Only in TGY-QAC broth.

TABLE 2. *Biochemical characteristics of* P. aeruginosa *strains QRM-S, QRM-R, and QRM-RN*

Character	Strain		
	QRM-S	QRM-R	QRM-RN
Pigment production			
Pyocyanin	+	O	+
Fluorescein	+	O	O
Hydrolysis of			
Milk	+	O (14 da)	S (14 da)
Gelatin	+	S (14 da)	S (7 da)
Aesculin	O	O	O
Acetic acid from ethanol	O	O	O
Oxidation of calcium lactate	+	+	+
Cytochrome oxidase	+	+	+
Gluconate oxidation	+	+ (slow)	+ (slow)
Catalase	+	+	+
Deamination of acetamide	+ (2 da)	+ (4 da)	+ (2 da)
Urease	O	O	O
Arginine dihydrolase	+	S[a]	S[a]
Proteinase	+	O	NR
Lipase	+	O	O
Dihydroxyacetone accumulation	O	O	O
Kosers citrate	+	S	S
Starch hydrolysis	O	O	O
Voges-Proskauer	O	O	O
Nitrate reduction	+	O	S
Lecithinase	+	O	O

+ = Strong positive, S = Weak positive, O = Negative, NR = Not recorded

[a]Medium turned blue faster than in negative controls

Electrophoresis

The total protein bands from sensitive and resistant cell sonicates were not identical. Furthermore, two distinct esterase bands were present in sensitive cells while the sonicates of resistant cells displayed only one band.

Biochemical analyses

A summation of the results of the biochemical tests conducted on the organisms is given in Table 2. Some of the most important differences were in pigment production, lipase activity, proteolytic ability, gluconate oxidation, and lecithinase activity. The sensitive cells produced amounts of both pyocyanin and fluorescein pigments. This was true even in the presence of sub-lethal concentrations of QAC. The resistant strain failed to produce detectable levels of either pigment, even in the absence of QAC. Strain QRM-RN did not produce fluorescein, but did produce a distinct, blue, water-soluble pigment as compared to the green pigment of the sensitive strain.

DISCUSSION

The slower growth of resistant cells due to a 25% longer generation time can not be attributed to the direct effect of the presence of QAC. It may be seen in Fig. 2 that resistant cells (QRM-RN) maintained 7 months in the absence of QAC exhibited a similar retarded growth rate. It was considered several years ago by Roberts and Rahn (*27*) that sublethal QAC concentrations may interfere with cell division. Perhaps an alteration to a less efficient metabolism could account for differences in growth rates. Accordingly, the protease activity of sensitive culture sonicates was much greater than sonicates of resistant cultures. Further study revealed the failure of resistant cultures to proteolyze milk or gelatin in 1 week while sensitive cultures displayed extensive proteolysis in 48 h. It may be reasoned from this that certain extracellular or cell surface enzymes were deficient in the resistant strain causing the culture to grow at a slower rate and fail to exhibit some of the biochemical properties possessed by its sensitive parent strain. Knox et al. (*17*) have previously reported inhibition of several enzyme systems by QAC and Stedman et al. (*33*) reported cell lysis by surface active agents to be secondary to enzyme inhibition in their mechanism of action. Exposure of sensitive cells to low levels of QAC has, to the contrary, been shown to stimulate enzyme systems (*29*).

Perhaps the major effect is on the cell envelope rather than on enzyme systems per se. Hotchkiss (*15*) demonstrated that QAC altered the cell membrane of at least part of a cell population. This report was later confirmed by Salton (*28*). Baker et al. (5) also suggested that QAC caused cell membrane disorganization and denaturation of certain proteins essential for metabolism

and growth. The initial results from permeability studies for this paper (Fig. 3) were somewhat diminished with later findings (*36*) that the resistant cell is considerably smaller than the sensitive cell. However, there do appear to be some cell forces involved which tend to lend support to Fig. 3. For example, washed cell suspensions of sensitive and resistant cells in distilled water had pH values of 6.7 and 7.4, respectively. This suggested differences in the net charges on the cell envelope and possibly alteration of destruction of cell membrane-oriented enzymes involved in transport systems. In addition, sensitive cells were easily lysed by washing five times in distilled water while resistant cells were resistant to this type of osmotic disintegration. Similarly, QAC-resistant cells of *E. coli* have an increased resistance to sonic disintegration and to surface active agents (*24*).

The death curve (Fig. 1) is of added interest since the recommended use concentration of QAC is 200 ppm (*34*). The maximum tolerance attained in a similar study (*24*) with *E. coli* in nutrient broth was 28 ppm. If cell lysis is one of the factors in the decrease of the sensitive population the difference between the two strains would be further magnified. Lysis of part of a cell population affords protection to the remaining cells by inactivating part of the QAC (*35*).

Maxcy et al. (*24*) reported that the maximum attainable viable population of QAC-resistant *E. coli* cells in nutrient broth containing 25 ppm QAC was 3.0×10^7/ml compared to 9.8×10^8/ml for the sensitive culture. Although a valid comparison can't be made because of the different genera and media employed, it is interesting to speculate on the differences in growth curve data obtained in this study (Fig. 2) and the data reported with *E. coli.* It might suggest that the presence of QAC is responsible for low maximum population attained with QAC-resistant *E. coli,* but further study would be required to support this speculation.

The reduced esterase and lipase activity of QAC-resistant cells indicated that the resistant cells were impaired in lipid catabolism, at least to the extent that they are unable to hydrolyze certain esters which accumulated in the medium. Since the esters detected were quite volatile, it is unlikely that they are important structural components of resistant cells causing the reduced permeability observed. The fruity aroma associated with broth cultures of the resistant strain was more noticeable in cultures grown on a shaker or sparged with air than in sationary cultures; the sensitive culture had the usual displeasing odor of *P. aeruginosa* with no traces of fruitiness. Furthermore, the presence of ethyl acetate and ethyl valerate in greater concentration in the resistant culture, as well as other unidentified compounds, also emphasizes the differences in the metabolic activity of the two strains, and since resistant cells contained 48% more total lipid than sensitive cells, involvement of lipids and lipid metabolism in QAC resistance was suggested. A role of lipids in resistance was proposed by Dyar and Ordal (*12*) and was given support by findings of Chaplin (*9*) and MacGregor and Elliker (*21*). Anderes et al. (*4*) recently made an extensive comparison between the lipids of QAC-resistant and -sensitive cells of *P. aeruginosa.* The possible involvement of acetate and acetyl CoA in the effects of QAC on the growth rate and lipid accumulation was discussed.

Several other changes have been observed in the transition from sensitive to resistant including the loss of flagella (*36*). Since it was not possible to classify the emerging QRM-R strain as *P. aeruginosa* using the flagella stain and biochemical tests, the possibility had to be considered that it was a contaminant introduced during transfers to acquire QAC resistance. The tests to support the identity of QRM-R were initiated by comparing thermal melting values of the isolated DNA from sensitive and resistant cultures; both strains had T_m values of 98 C, in agreement with reported values (*22*). In addition, *P. aeruginosa* phage 4 lysed both the sensitive and resistant strains. The typing of *P. aeruginosa* with phage was suggested by Postic and Finland (*26*). Further support could have been provided by pyocin typing (*14*) or by immunotyping; neither of which is completely reliable (*2*). However, the only species known to excrete pyocyanin is *P. aeruginosa* (*3*) and, fortunately, it was observed that QRM-R cells grown in the absence of QAC for extended periods, designated QRM-RN, regain the ability to produce pyocyanin. This, coupled with the positive indophenol (cytochrome) oxidase test (*13*), provides a sound basis for identification of the QRM-R strain as a descendant of the original *P. aeruginosa* culture.

It is reasonable to expect that sub-lethal concentrations of QAC have physical and chemical effects on sensitive cells. Being a surface active cationic detergent, QAC would have affinity for cellular lipid. If this were the sole effect of QAC, one would not expect retention of resistance after growth in the absence of QAC or after washing the cells. Therefore, it seems that QAC penetrates the cell envelope and acts on some area inside the cell controlling lipid metabolism. QAC may destroy the sensitive cells in a population and select for the resistant cells or it may actually induce mutations in sensitive cells converting them to resistant cells. It is apparent that the presence of QAC in the growth medium causes some changes that last only as long as QAC is present. However, other changes last through hundreds of generations in the absence of QAC. This suggests that the action of QAC is many-fold. There is the effect on membrane integrity due to its surfactant nature; the effect as a cationic detergent on membrane-oriented enzyme systems; and, its effects once inside the cell, both on lipid metabolism and on the protein synthesizing machinery of the cell, including DNA.

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Estimating Population Levels of *Clostridium perfringens* in Foods Based on Alpha-toxin

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ABSTRACT

A method for estimating population levels of *Clostridium perfringens* in foods based on the titer of *a*-toxin in food extracts was evaluated. Twenty-two samples of food associated with 15 food poisoning outbreaks and 12 ready-to-serve foods inoculated with approximately 100 *C. perfringens* spores/g and incubated to simulate improper holding conditions were examined. Alpha-toxin was present in extracts from most of the foods which could be correlated with the viable population as determined by plate counts in sulfite-polymyxin-sulfadiazine (SPS) agar. The amount of *a*-toxin present could be correlated with previous growth of *C. perfringens* in food regardless of whether the organisms were viable when the examination was made. This is important because storage and shipment of the food specimens for brief periods at low temperature results in a decrease of 2 to 4 log cycles in the viable plate count in SPS agar, but does not affect the accuracy of estimates of the population level based on the presence of *a*-toxin.

In many countries, including the United States, *Clostridium perfringens* is now widely recognized as one of the principal causes of food poisoning of bacterial origin (*3, 6, 10*). The illness occurs upon ingestion of foods containing large populations of viable vegetative cells of *C. perfringens* and the subsequent production of an enterotoxin in the intestine (*9, 11*). The rapid loss of viability of this organism, which occurs when food samples associated with food poisoning outbreaks are frozen for storage or shipment to the laboratory, has led to much difficulty in firmly establishing *C. perfringens* as the cause of many outbreaks. A decrease of 2 to 4 log cycles in viable plate counts of this organism is not uncommon when food samples are stored at low temperature for only a few days (*2, 7*). Therefore, cultural methods used for quantitation of this organism are often inadequate for an accurate determination of population levels in outbreak foods.

A method for estimating population levels of *C. perfringens* in food, which utilizes the hemolytic and lecithinase activities of *a*-toxin (phospholipase C) produced by this organism as an index of growth, was developed earlier in our laboratory (*7, 8*). The purpose of this study was to further evaluate the utility of the *a*-toxin method for the examination of a large variety of different foods associated with foodborne disease outbreaks. It should be understood that *a*-toxin and the enterotoxin produced by *C. perfringens* type A are different toxins. The enterotoxin which is responsible for gastroenteritis in outbreaks of food poisoning due to *C. perfringens* is produced only after ingestion of the contaminated food and is not usually present in the incriminated food.

MATERIALS AND METHODS

Examination of outbreak foods

A variety of foods associated with 15 food poisoning outbreaks which were attributed to *C. perfringens* were examined. All of the foods were supplied by laboratories of various state and municipal health departments who were conducting the investigation of the different food poisoning outbreaks. The incriminated food samples were collected from the cooperating laboratories by personnel from the District Laboratories of the Food and Drug Administration or the Center for Disease Control in Atlanta, Ga., and shipped frozen with dry ice to our laboratory. A few samples were sent directly to us by the cooperating laboratories.

The samples were stored frozen and examined within 18 to 24 h for viable *C. perfringens* by plate counts in sulfite-polymyxin-sulfadiazine (SPS) agar and for the presence of *a*-toxin by the method described previously (*7*). Of the various foods examined, eight consisted of roast beef, three of beef juice, three of turkey, two of beef hash, two of beef gravy, and one each of sliced ham, pork barbeque, chicken salad, and clam sauce. Most of the foods tested had been shown to contain a large viable population of *C. perfringens* by the laboratory that initially investigated the outbreak.

Examination of inoculated foods

The 12 ready-to-serve foods used for the temperature abuse experiments were all obtained from local food service establishments. The various foods tested included: roast beef, ground beef, meat loaf, beef hash, turkey, beef gravy-mix, chicken salad, sliced pork, and crabmeat. Between 100 and 200 g of the food were placed in sterile plastic bags and either inoculated directly with *C. perfringens* spores or immersed in a water bath for 2 h at 50 C to reduce oxygen levels in the food before inoculation. Each food was inoculated with approximately 100 spores/g of one of the strains studied. As much air as possible was evacuated from the bags, which were heat sealed and incubated at 35 C.

Foods were examined periodically during incubation for evidence of the growth of *C. perfringens*. When large populations of *C. perfringens* were present in foods, as indicated by gas production in bags, or by direct microscopic examination, a portion of each sample was examined for viable *C. perfringens* by plate counts in SPS agar and for *a*-toxin by the method previously described. The remainder of each food was frozen and stored for 3 weeks at -20 C and reexamined for viable *C. perfringens* and for *a*-toxin.

Spore stocks

All of the *C. perfringens* strains used in these experiments were isolated from food or feces associated with foodborne disease outbreaks. Strains FD-1, FD-2, and FD-26 were isolated in our laboratory. Strains CDC 1861 and CDC 2078 were obtained from V. R. Dowell, Center for Disease Control, Atlanta, Ga. Strains A-86 and S-34 were obtained from H. E. Hall, U.S. Public Health Service, Cincinnati, Ohio. Strains NCTC 8797, NCTC 8238, NCTC 8798, and NCTC 10240 were supplied by the National Collection of Type Cultures, London, England.

The spore stocks used for inoculation of the ready-to-serve foods were grown in either Ellner's medium (*5*) or in Duncan-Strong spore broth (*4*). Fifteen-milliliter volumes of each spore broth were inoculated with 2 ml of an 18-h fluid thioglycollate culture of each strain and incubated at 35 C for 24 to 48 h. The spores were harvested by centrifugation, resuspended in sterile water, and stored at 4 C.

Extraction and quantitation of a-toxin

The procedures for extracting and quantitating *a*-toxin in foods were described previously (*7*). These procedures were followed throughout the present study except that the supernatant extract was filtered with Whatman No. 1 filter paper before Seitz filtration. Removal of fat and particulate material by filtration with paper greatly facilitated filter sterilization of the extract. Extracts from turkey samples and the chicken salad sample also required treatment with ethyl ether to remove lipids before filter-sterilization of the extract, but this step was not necessary for any of the other foods. Details of this procedure were presented in a previous publication (*7*).

Viable counts

A 25-g portion of food was blended with 225 ml of 0.1% peptone water, diluted serially in the same diluent, and plated in laboratory-prepared SPS agar (*1*). After solidification, this agar was overlaid with an additional 5 ml of SPS agar and incubated for 24 h in a Case Anaero-jar (Case Laboratories Inc., Chicago, Ill.) under an atmosphere of nitrogen or in Gas Pak jars (BBL, Cockeysville, Md.). Black colonies were counted, and 10 isolates were picked to motility-nitrate medium for confirmation as *C. perfringens*.

Correlation of viable count with a-toxin

The relationship between viable plate counts in SPS agar and the amount of *a*-toxin produced in a limited number of foods has been established (*7*). Data obtained with six strains of *C. perfringens* isolated from outbreak foods (chicken broth) were chosen as the basis for correlating population levels with the *a*-toxin titer. The *C. perfringens* strains used for the determinations in chicken broth included three strains which produce heat-sensitive spores (FD-1, CDC 1861, and S-34) and three strains with heat-resistant spores (NCTC 8238, NCTC 8797, and NCTC 8798). Two-hundred grams of chicken broth were heated to expel oxygen and inoculated with approximately 5×10^5 washed cells/ml of the test strain and incubated at 35 C. After 2 h of incubation, samples were removed at 30-min intervals and tested for *a*-toxin and plate counted. Alpha-toxin titers were determined in the hemolysin indicator plate and the lecithovitellin (LV) test as previously described (*7*). The geometric mean value of the viable counts of the six strains was determined and correlated with the corresponding *a*-toxin titer for each population level.

RESULTS

Correlation of viable count with a-toxin

The relationship between viable plate counts of *C. perfringens* in SPS agar and the amount of *a*-toxin produced in a limited number of foods has been established (*7*). Since experimental results obtained with roast beef and chicken broth were similar, data from experiments with six representative "food poisoning" strains of *C. perfringens* in chicken broth were chosen as the basis for correlating population levels with the amount of *a*-toxin present in foods. For easy reference in interpreting the results of the present report, these data are presented again in Table 1.

TABLE 1. *Correlation between the viable count of* Clostridium perfringens *and the amount of a-toxin detected in filtrates of chicken broth*

Viable count/g[a]	Alpha toxin titer: Hemolysin indicator plate	Alpha toxin titer: Lecithovitellin test
1.2	Undiluted	No reaction
2.5	1:2[b]	Undiluted
6.5	1:4	1:2[c]
9.5	1:8	1:4
25	1:16	1:8
55	1:32	1:16
80	1:64	1:32
150	1:128	1:128
210	1:256	1:256

[a]Average of viable counts obtained with six strains. Values to be multiplied by 10^6.
[b]Dilution of extract producing a 1-mm zone of hemolysis.
[c]Highest positive dilution.

These results show the relationship between the average plate count in SPS agar and the corresponding *a*-toxin titer produced in chicken broth as measured in hemolysin indicator plates and the LV test. Data in Table 1 were used throughout the study for estimating population levels in the various outbreak foods and in the inoculated ready-to-serve foods.

Viable counts and population estimates with outbreak foods

A total of 22 food samples associated with 15 food poisoning outbreaks were examined. The viable plate counts of *C. perfringens* determined before and after

TABLE 2. *Comparison of viable count with an estimation of the previous growth based on the quantity of a-toxin detected in foods associated with foodborne disease outbreaks*

Food	Viable count per g: Before freezing[a]	Viable count per g: After frozen storage[b]	Alpha toxin titer in hemolysin indicator plate	Estimated population/g based on *a*-toxin titer
Beef prime ribs[c]	1.0×10^7	1.2×10^6	1:8	9.5×10^6
Roast beef[c]	5.5×10^6	5.0×10^4	1:4	6.5×10^6
Roast beef[c]	1.4×10^3	1.0×10^2	1:2	2.5×10^6
Roast beef	ND[d]	2.1×10^4[d]	0	$< 10^6$
Roast beef	ND	3.0×10^4	0	$< 10^6$
Barbecue beef hash[c]	6.3×10^7	1.5×10^3	1:64	8.0×10^7
Beef hash[c]	9.0×10^4	10	1:2	2.5×10^6
Beef juice	3.0×10^7	2.7×10^5	1:16	2.5×10^7
Beef juice	1.2×10^7	-7.0×10^4	1:8	9.5×10^6
Beef gravy	3.5×10^7	2.3×10^5	1:16	2.5×10^7
Ham	3.0×10^7	4.7×10^3	1:32	5.5×10^7
Barbecue pork	1.0×10^7	3.0×10^2	1:4	6.5×10^6
Chicken salad	4.0×10^5	5.0×10^2	conc. × 2	5.0×10^5
Turkey	ND	1.0×10^2	undiluted	1.2×10^6
Turkey salad	5.3×10^6	2.1×10^3	undiluted	1.2×10^6
Clam sauce	5.0×10^5	5.0×10^2	undiluted	1.2×10^6

[a]Data supplied by the laboratory conducting investigations of the outbreak.
[b]Data obtained in FDA laboratory after frozen shipment of the food.
[c]Previously published data (*7*).
[d]No data available.

frozen shipment and storage and estimated population levels based on the quantity of *a*-toxin present which were obtained with 16 representative outbreak foods are presented in Table 2. Data obtained with some of the outbreak foods has been reported previously (*7*), but are included in Table 2 because they were more typical of the outbreak foods examined than those for which no data are presented. Similar results were obtained with the remaining six food samples examined. Plate counts with these foods ranged from 1.4×10^3 to 3.5×10^7/g before they were frozen for shipment to our laboratory. Alpha-toxin was present in 14 of these foods which could be quantitated and utilized for estimating the population level of *C. perfringens*.

Population estimates were based on the titer of *a*-toxin in hemolysin indicator plates and *a*-toxin present identified by neutralization with *C. perfringens* alpha antitoxin. Identity of *a*-toxin in food extracts was also considered to be more firmly established if lecithinase activity of extracts in the LV test was prevented by addition of *a*-antitoxin. There was very good agreement between the plate counts obtained in other laboratories before frozen shipment of the samples and the estimated population level based on *a*-toxin with most of the outbreak foods. The estimated population levels ranged from $<10^6$/g to 5.5×10^7/g and most instances were within 1 $\log_{10}$ of the plate count reported by the original investigators. However, after frozen shipment of the food samples, plate counts in SPS agar were reduced substantially and therefore were unreliable for determining population levels because of the loss of viability of *C. perfringens* in the incriminated foods. A decrease of 2 to 4 log cycles in the plate count in SPS agar usually occurred during storage and frozen shipment to our laboratory.

With two samples, the estimated population level based on *a*-toxin was substantially higher than the original plate counts indicating that the viable population may have declined before the plate counts were made. Alpha-toxin was not detected in any of the foods which had a population level less than 5.0×10^5/g.

Viable counts and population estimates in inoculated foods

In addition to the outbreak foods, samples of 12 ready-to-serve foods which were inoculated with 100 *C. perfringens* spores/g and incubated to simulate improper storage conditions were examined. The viable plate counts in SPS agar and estimated population levels based on the titers of *a*-toxin found in extracts of the inoculated foods are shown in Table 3. Immediately following incubation, a large population of *C. perfringens* was present in all of the foods tested. Viable counts in SPS agar ranged from 3.0×10^6/g in roast beef inoculated with strain NCTC 8798 to 2.2×10^8/g in beef hash inoculated with strain FD-1. Detectable *a*-toxin was also present in all of the foods which could be quantitated and correlated with the viable plate counts in SPS agar with the exception of roast beef inoculated with strain NCTC 8798. Storage of the inoculated foods for 3 weeks at -20 C resulted in a substantial decrease in the viable plate counts in SPS agar as shown in Table 3. The viable counts decreased from 4.5×10^7/g to 3.4×10^3/g during frozen storage of a sample of roast beef inoculated with strain FD-1. A similar result was obtained with many of the other foods.

The *a*-toxin titer present in extracts from the various foods remained unchanged during the 3-week storage period. Therefore, only the results obtained after frozen storage are presented. The estimated population levels based on the titer of *a*-toxin present in food extracts were virtually identical with plate counts obtained in SPS agar with six of the inoculated foods. However, the estimated population levels obtained with foods inoculated with strains NCTC 8238, NCTC 10240, and FD-26, which produce less *a*-toxin, were lower than the viable plate count by a factor of 2.5 to 16 times. One sample of roast beef inoculated with strain NCTC 8798 was negative for *a*-toxin but had a plate count indicating a population of 3.0×10^6/g.

DISCUSSION

Results obtained with most of the outbreak foods and

TABLE 3. *Comparison of viable count with an estimation of growth based on the quantity of a-toxin detected in a variety of foods inoculated with spores of* Clostridium perfringens[a]

Food	Strain	Viable count per g		Alpha-toxin titer in hemolysin indicator plate[b]	Estimated population/g based on the *a*-toxin titer
		Before freezing	After 3 wk of frozen storage		
Roast beef	FD-1	4.5×10^7	3.4×10^3	1:64	8.0×10^7
Roast beef	NCTC 8798	3.0×10^6	1.6×10^4	0	$< 10^6$
Meat loaf	NCTC 8797	9.0×10^7	9.3×10^4	1:256	2.1×10^8
Beef hash	FD-1	2.2×10^8	9.1×10^6	1:128	1.5×10^8
Ground beef	FD-2	1.9×10^8	6.9×10^4	1:256	2.1×10^8
Ground beef	CDC 1861	1.7×10^8	1.1×10^5	1:128	1.5×10^8
Ground beef	NCTC 8238	1.6×10^7	2.1×10^5	1:2	2.5×10^6
Turkey	A 86	2.8×10^7	7.2×10^3	1:16	2.5×10^7
Beef gravy mix	NCTC 10240	4.0×10^7	5.5×10^4	1:2	2.5×10^6
Chicken salad	FD-26	2.3×10^7	8.7×10^3	1:8	9.5×10^6
Sliced pork	CDC 2078	1.9×10^8	4.4×10^5	1:256	2.1×10^8
Crab meat	S-34	3.0×10^7	2.8×10^4	1:32	5.5×10^7

[a]Foods were inoculated with approximately 100 spores per gram and incubated for 18-48 h at 35 C.
[b]Alpha-toxin titers were determined after 3 weeks of storage at −20 C.

inoculated ready-to-serve foods showed that there was a reasonably good correlation between the viable population of *C. perfringens* and the estimated population level based on the titer of *a*-toxin found in the food extracts. These results support our previous findings that in most cases detectable *a*-toxin is present in extracts from foods containing populations of *C. perfringens* greater than 10^6/g which can be utilized to estimate population levels when the organisms themselves are no longer viable. Data obtained with a variety of outbreak foods naturally contaminated with *C. perfringens* and with food inoculated with spores and incubated to simulate improper holding of foods, indicate that population estimates based on the titer of *a*-toxin in food extracts are usually quite reliable.

False-negative results were obtained occasionally with this method due to insufficient *a*-toxin production by a few strains. This finding was anticipated from our experimental results which showed that 10 of 34 strains of the *C. perfringens* isolated from outbreak foods produced less than average amounts of *a*-toxin and that 2 of the 34 strains tested were poor *a*-toxin producers (*7*). However, we considered this shortcoming to be outweighed by the potential usefulness of the method. As the data in Tables 2 and 3 show, the estimated population levels based on the titer of *a*-toxin in food extract were much more indicative of the actual *C. perfringens* population than were viable plate counts made after storage and shipment of the foods at low temperature. Since this is the usual practice in handling food specimens for bacteriological examination, plate count determination of *C. perfringens* populations in foods shipped or stored at low temperature are likely to be highly inaccurate.

The type of food associated with the different outbreaks appears to have had little effect on population estimates based on the presence of *a*-toxin. Results obtained with the outbreak foods suggest that whenever conditions were suitable for growth of *C. perfringens* *a*-toxin was usually produced. It appears that the quantity of *a*-toxin detected in food extracts depended more on toxin-producing ability of the strains present than any other factor. In a few cases when population levels were near the lower limits for production of detectable *a*-toxin, no toxin was found in the food extracts.

The stability of *a*-toxin in foods stored for prolonged periods at 4 C or below and the relative ease with which it can be extracted and quantitated provide an additional means for estimating large populations of *C. perfringens* in foods. The population estimates, while not strictly quantitative, should be valuable for identifying foods responsible for *C. perfringens* food poisoning outbreaks and determining the hazard presented by the development of large populations of this organism in foods.

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A Microbiological Survey of Three Fresh Green Salads- Can Guidelines be Recommended for these Foods?

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ABSTRACT

One hundred samples each of mixed green salad, green salad, and coleslaw were selected from two centralized food service divisions of large military hospitals. Samples were obtained 5 days per week in accordance with production schedules, and were analyzed for total plate count, total coliforms, both plate and Most Probable Number Methods, *Escherichia coli,* and *Staphylococcus aureus.* Extremely wide ranges were found in total plate counts and total coliform counts; while confirmed *E. coli* and *S. aureus* counts were quite low. The feasibility of establishing microbiological guidelines for these products is discussed.

In the past few decades, the food service industry had undergone a process of modernization and streamlining. Labor saving methods with associated monetary savings have become a necessity to be competitive in the modern food industry.

Concurrently with changes in the food service industry, interest in microbiological standards and guidelines has increased. The consuming public has shown interest in the microbiological quality of food products, and has been appalled by the "high" numbers of microorganisms quoted by uninformed news media and misleading reports of consumer protection agencies. The natural flora of good quality foods, particularly those of plant origin, often has been overlooked, and thus a false image of product quality presented to the public. Our interest recently has focused upon the microbiological quality of fresh green salads prepared for serving by a modern central food preparation facility (*6*). The purpose of the present study was to determine the feasibility of developing control guidelines on the quality of the finished products offered for serving.

MATERIALS AND METHODS

Samples

Three products—mixed green salad, green salad, and coleslaw (without dressing), were selected for study. The mixed green salad was composed of lettuce, radishes, and tomatoes; the green salad was fresh lettuce; and the coleslaw contained shredded cabbage. Two centralized food service divisions of large military hospitals were chosen as sources of supply for samples because of proximity to the laboratory and for the high degree of sanitation maintained in their operations. Samples of approximately 100 g were collected in sterile polyethylene bags immediately before placing individual portions on the serving line. Due to production schedules, each product was not necessarily sampled daily, but samples were obtained 5 days per week. One hundred samples of each type of salad were analyzed.

Sample preparation

A 25-g sample was weighed into a sterile 1-liter stainless steel blender cup and 225 ml of sterile buffered water (*3*) added. The sample was blended for 3 min at high speed. Consecutive serial dilutions to 10^{-7} were prepared and the following analyses performed.

Total plate count

Duplicate plate, 10^{-1} thru 10^{-7} (higher if necessary) were prepared, poured with 12-14 ml of Standard Methods Agar, allowed to solidify, and incubated at 32 C for 72 h. Preparation, counting, and reporting were done in accordance with *Standard Methods for the Examination of Dairy Products* (*3*).

Total verified coliform count, plate method

Duplicate plates, 10^{-1} thru 10^{-5} dilutions, were prepared, poured with 10-12 ml of violet red bile (VRB) agar and overlaid with 3-4 ml of VRB. After solidifying, plates were incubated at 32 C for 24 h. Colonies with typical coliform morphology (for VRB) were counted. When present, coliforms were verified by transferring at least five colonies per plate to lauryl sulfate broth (LSB) dispensed in 10-ml amounts in test tubes containing fermentation vials. These were incubated at 32 C for 48 h. Tubes showing positive reactions were then transferred to brilliant green bile (BGB) broth. The percentage of tubes showing visible gas in the fermentation vial after incubation at 32 C for 48 h was determined, multiplied by the presumptive count, and the results reported as the verified total coliform count.

Total coliform count, MPN method, and the fecal coliform count

A three-tube MPN series was prepared and calculated in accordance with *Reference Methods for the Microbiological Examination of Foods* (*2*). The MPN was calculated from a standard MPN table on the basis of the confirmed results (*2*).

Staphylococcus aureus, MPN

A three-tube MPN series was prepared in accordance with the AOAC method (*1*) *except* that isolation was accomplished on tellurite polymyxin-B egg yolk (TPEY) agar. The tube coagulase test (*1*) was done, as necessary, on isolates from TPEY agar plates.

RESULTS

Total plate counts (TPC) (Fig. 1) of the three items selected showed a similar pattern. The TPC of mixed green salad ranged from $3.7 \times 10^3 - 7.3 \times 10^8$/g with a mean of 1.6×10^7/g; TPC of green salad ranged from $3.0 \times 10^3 - 3 \times 10^7$/g, while its mean was 1.9×10^7/g; coleslaw TPC ranged from $3.0 \times 10^6 - 1.6 \times 10^8$/g, with a mean of 4.7×10^6/g. The distribution of counts for the three items showed a remarkedly similar pattern.

Coliform plate counts (Fig. 2) exhibit similar patterns for the three types of salads. The range for mixed green was $1.0 \times 10^1 - 2.1 \times 10^5$/g, for green salad, $1.0 \times 10^1 - 1.6 \times 10^5$/g, and $1 \times 10^1 - 8.5 \times 10^4$/g for coleslaw. The

mean values for mixed green, green, and coleslaw were 5500/g, 3600/g, and 2200/g, respectively.

As is inherent in the MPN procedure, coliform MPN (Fig. 3) results were higher than the coliform plate

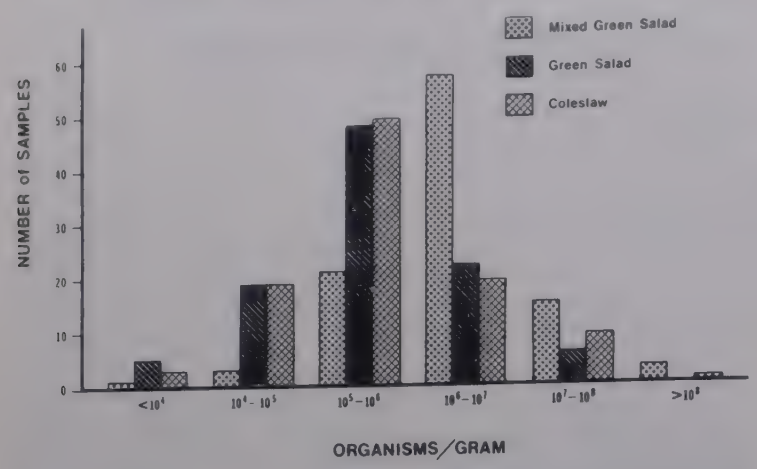

Figure 1. *Total plate counts in mixed green salads, green salad, and coleslaw.*

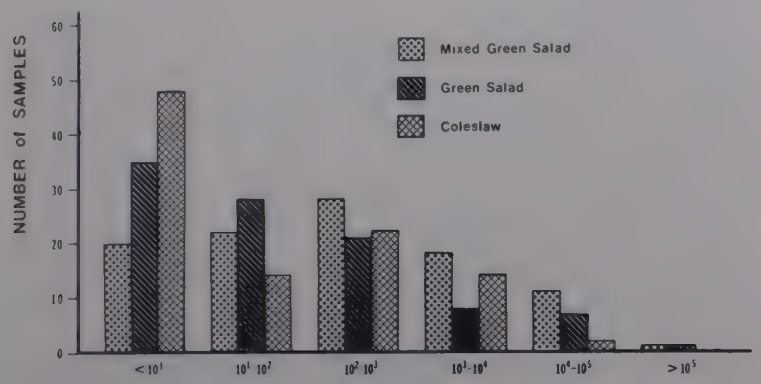

Figure 2. *Total coliforms (plate method) in mixed green salad, green salad, and coleslaw.*

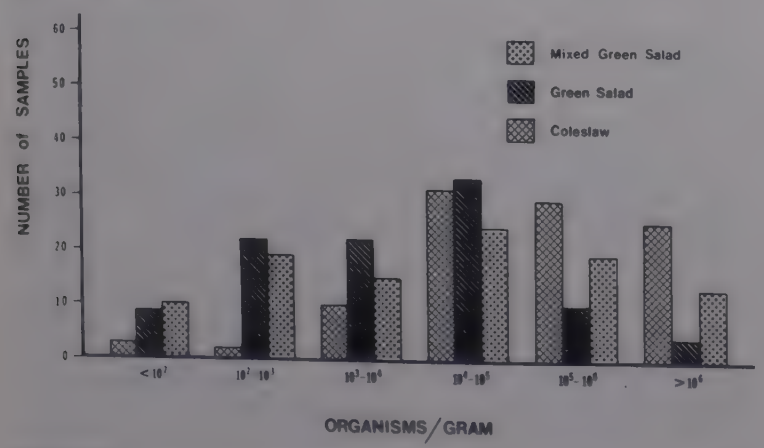

Figure 3. *Total coliforms (Most Probable Number method) in mixed green salad, green salad, and coleslaw.*

counts. The mean values for mixed green salad, green salad, and coleslaw were 14,000/g, 79,000/g, and 49,000/g, respectively.

Twenty-six percent of the mixed green salads, 28% of the green salads, and 29% of the coleslaw samples contained fecal coliforms (see Fig. 3). The IMViC confirmation for *E. coli*, however, was 3%, 2%, and 6% for mixed green, green, and coleslaw, respectively.

S. aureus was found in 8% of the mixed green salads, in 14% of the green salads, and in 3% of the coleslaw samples. The highest counts were 2400/g in mixed green salad, and 1400/g in green salad.

DISCUSSION

Many different types of microorganisms have been reported to be normal inhabitants of plants, or commonly associated with plant products. Mundt (*9*) has extensively investigated the presence of lactic acid bacteria, and reported that few species are consistently found on plants. In addition, he showed that many species required special conditions for enumeration, and that the number of organisms/g was highly variable. Samish, et al. (*11*) have shown that microorganisms are present within the tissue of healthy tomatoes, and that their frequency between different fields of tomatoes is variable. In his work, Splittstoesser (*13*) has emphasized the extreme variability of counts between different vegetables, and found that those physically protected (as by pod or husk) may possess counts as high as those grown in direct contact with the environment.

The isolation of pathogens from vegetable material is documented in the literature. Green et al. (*7*) demonstrated that *Pseudomonas aeruginosa* may be isolated from a small percentage of fresh vegetable samples, while Dunlap and Wang (*5*) isolated viable *Salmonella* in 1 of 97 samples grown on land irrigated with sewage effluent. Brown and Seidler (*4*) found nearly 50% of vegetables analyzed to be contaminated with *Klebsiella pneumoniae.* Many investigators have reported numerous isolations of *Escherichia coli, Staphylococcus aureus,* and other pathogens from plant material. In an indepth review, Pasch (*10*) has shown that nosocomial infections can readily occur from and through food. He postulated that proper food handling and education are the best methods for controlling nosocomial infections. It is important to note that microbiological guidelines were not considered to be the most effective for control, even in a situation where debilitated persons were the population at risk.

Evidence seems to indicate that the microbial population of vegetable products increases as the direct result of processing (*12*). These investigators observed that manipulation distributes the initial bacterial flora of vegetables, as well as supplements the initial flora, and results in a product with a higher bacterial population. In working with prepackaged salad greens, they found that the microbial population increased with the length of storage time and with an increase in moisture.

Results of the present study tend to reinforce the previously reported variability in bacterial load between samples; however, our results show a uniformity between products when speaking of the total plate count (Fig. 1). When one considers the range of total plate counts found in analyzing good quality products, the feasibility of imposing microbiological guidelines appears remote. When other factors (seasons of the year and different locales of production) are considered, the application of guidelines appears inadvisable.

In this study, total coliforms were determined by both the plate count and the MPN methods. As is to be expected, the MPN results were considerably higher than the plate counts. This observation reinforces the findings of other workers (*8*) who have proposed the use of the MPN techniques rather than plate counts in analyzing foods other than dairy products. As with total plate count, extreme variability was found between individual salads, and considerable differences were observed in total coliform counts between types of products.

The percentages of salads which were positive for fecal coli in EC broth were remarkedly similar. Confirmation of *E. coli* by biochemical methods, however, was extremely low.

S. aureus counts (MPN method) were extremely low, and were probably due to contamination by food service personnel rather than as natural plant inhabitants.

In considering the basic purpose of this study (the feasibility of establishing microbiological guidelines), the authors conclude that the extreme variability of analyses within a given product negate this possibility. Neither the total plate counts nor the total coliform counts exhibit enough uniformity to recommend this action. If special applications, such as hospital feeding or for special patient feeding are intended, then special procedures of analysis for all pathogens could be done. For routine applications, however, establishment of microbiological guidelines for the three products in this study (mixed green, green salad, and coleslaw) appear to be unnecessary.

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Evaluation of Diluents Used for Total Counts[1]

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ABSTRACT

A comparison was made between Butterfield's buffered phosphate solution, 0.1% peptone in distilled water, 0.5% peptone in distilled water, 0.85% NaCl in distilled water, and pure distilled water as diluents for total bacterial counts of 28 different food samples. Plates were incubated at 35 and 22 C for 2 and 5 days, respectively. Butterfield's diluent afforded the highest overall mean count for most samples regardless of the incubation temperature. Highest counts occurred with all diluents at 22 C. Although Butterfield's diluent, 0.1% peptone, and 0.5% peptone appeared to yield higher counts for most samples than did distilled water or 0.85% NaCl, statistically the mean counts obtained for the five diluents used were not significantly different from each other. It appears that no single diluent is suited for every possible sample type.

The reliability of various diluents used in routine microbial analyses has been questioned and investigated by many workers. Most investigations have been concerned with survival of specific bacteria or mixed populations of bacteria in different diluents for various time periods (*1, 2, 5, 7, 9, 10*). Of most interest for routine bacterial counts are those studies involving mixed populations of bacteria such as occur in most food samples. Notable among these investigations was Butterfield's work (*2*) on survival time of bacteria in water samples. Butterfield suggested the use of phosphate buffer and/or other minerals for most consistent results. Peptone added to distilled water was tested as a diluent by Straka and Stokes (*7*) and proved to have advantages over distilled water or phosphate diluent for bacteria recovered from precooked frozen chicken and turkey pies. Patterson and Cassells (*5*) found that small amounts of peptone added to such diluents as 4% NaCl, 20% NaCl, and quarter stength Ringer's solution slightly decreased bacterial destruction in analysis of bacon curing brines. Recently Huhtanen et al. (*4*) examined the use of phosphate buffer and distilled water dilution blanks for plate counts of raw milk bacteria and found that counts were higher in diluent without phosphate buffer.

The initial effect of diluents on the total counts of food samples was the primary concern in our study, since in routine bacterial analyses most samples are plated within 20 min or less. The purpose of this investigation was to evaluate five diluents for suitability in determining total bacterial counts of several food products using standard microbiological procedures.

[1]*Florida Agricultural Experiment Stations Journal Series No. 6040.*

MATERIALS AND METHODS

Samples

All 28 food samples were obtained from retail stores in Gainesville, Florida. Sixteen samples representing fresh, cooked, or smoked meats, six samples representing fresh or frozen vegetables, and three samples representing dairy products were analyzed.

Preparation of samples

A quantity of each sample large enough to yield five 50-g sub-samples was obtained. Each of the 50-g subsamples was blended with 450 ml of the appropriate sterile diluent for 2 min in a Waring blendor at 8,000 rpm. Additional dilutions were made by transferring 11 ml into 99 ml dilution blanks as needed (*8*). Two sets of duplicate plates for each dilution were prepared. All plates were poured with Standard Methods agar (Difco) 8 to 10 min after blending of the sample, and one set was incubated at 35 C for 48 h and the other at 22 C for 5 days. This procedure was followed with each of the five diluents. The pH of all diluents was monitored after autoclaving and cooling, and again after mixing with the samples.

The following diluents were used: 0.1% peptone in distilled water, 0.5% peptone in distilled water, Butterfield's buffered phosphate solution (*2*), 0.85% NaCl in distilled water, and distilled water.

Statistical methods

The Statistical Analysis System (SAS) (*6*) program was used to evaluate the data obtained. Means of counts for diluents on each sample, on several food groupings, and on all samples were made and compared using Duncan's 5% level new multiple range test. Also, rankings of diluents for each sample were made using Friedman's rank analysis of variance test for significant differences (*3*). Data from each incubation temperature and from both temperatures pooled were used for these analyses.

RESULTS AND DISCUSSION

Comparisons of bacterial recovery of diluents for each sample at 35 C are presented in Table 1. These data indicate that there were differences in the recovery of organisms with each of the diluents from sample to sample as would be expected from the diversity of bacterial populations in a variety of foods. Recoveries were always higher when incubation was at 22 C.

The combined results show no overall significant statistical differences between the recovery of organisms with each diluent, as might be expected with such variability from sample to sample (Tables 2 and 3). However, it is of interest to note that Butterfield's diluent had the highest overall means and rankings while distilled water and 0.85% NaCl had the lowest overall means and rankings with pooled data from both incubation temperatures. The pooled data from the meat samples yielded similar results, as would be expected since most

TABLE 1. *Effect of diluents[1] on recovery of bacteria for each sample at 35 C*

Sample	Mean log counts[2]				
Meats:					
	C	B	A	D	E
Bacon	3.6989	3.6155	3.5792	3.5492	3.3155
	B	E	A	D	C
Bologna	4.7682	4.6982	4.6320	4.5668	4.4668
	D	E	B	C	A
Frozen chicken thighs	6.4270	6.1105	5.9031	5.6812	5.4939
	C	B	E	A	D
Frozen chicken wings	4.0293	3.9747	3.9182	3.8323	3.8070
	C	A	B	D	E
Frozen fish sticks	4.9441	4.8745	4.6672	4.2763	4.2466
	C	A	E	D	B
Fresh sausage	7.2473	7.1626	6.9503	6.8125	6.7094
	C	E	B	D	A
Fresh fish (Turbot)	3.6902	3.6506	3.6021	3.1505	3.1505
	B	E	A	C	D
Frozen sausage	6.0969	6.0064	5.9717	5.5468	5.5434
	D	A	B	C	E
Frozen ground beef	6.3373	6.2785	6.1019	5.9638	5.8856
	A	D	C	B	E
Head cheese	8.6196	8.5629	8.4691	8.4675	8.4186
	B	C	E	A	D
Frozen liver	4.7500	4.6382	4.4388	4.3784	4.3559
	D	A	C	E	B
Liver cheese	6.5550	6.2623	6.9978	5.9835	5.8195
	A	D	E	C	B
Pork stew meat	5.6809	5.6738	5.4784	5.4698	5.3739
	C	A	D	B	E
Raw oysters	8.4305	8.3383	8.3335	8.2953	8.1709
	D	C	B	A	E
Salami	7.2237	7.2235	7.1536	7.1154	7.1021
	A	C	D	B	E
Wieners	3.9215	3.8737	3.8661	3.7442	3.6153
Vegetables:					
	D	B	C	A	E
Fresh broccoli	7.2393	6.7915	6.6173	6.5439	6.2884
	C	B	E	A	D
Fresh green beans	7.9762	7.7153	7.5227	7.4796	7.3355
	A	D	B	E	C
Frozen broccoli	5.1596	5.0984	5.0826	5.0769	5.0301
	A	D	E	B	C
Frozen green beans	3.7776	3.5573	3.5481	3.3873	3.3613
	D	E	A	B	C
Frozen lima beans	4.8646	4.6470	4.5880	4.3802	4.2912
	C	B	E	A	D
Frozen mixed vegetables	3.6127	3.5608	3.5563	3.3585	3.1515
	B	A	E	C	D
Beef pot pie	4.3139	3.9708	3.8747	3.8351	3.5924
	A	B	E	C	D
Chicken pot pie	3.2612	3.2380	3.1238	3.9742	2.8976
	C	D	E	A	B
Turkey pot pie	4.2261	4.1942	4.0942	4.0898	3.8997
Dairy Products:					
	E	C	D	B	A
Cheddar cheese	6.6580	6.1575	6.1301	6.1021	6.0051
	B	A	C	E	D
Blue cheese	6.5792	6.5716	6.4486	6.2776	6.2053
	A	C	E	B	D
Buttermilk	5.8268	5.7605	5.4741	5.4158	5.2188

[1]A = Butterfield's diluent, B = 0.5% peptone, C = 0.1% peptone, D = 0.85% NaCl, and E = Distilled H_2O

[2]Recoveries underscored by the same line are not significantly different at the 0.05 level.

TABLE 2. *Effect of diluents[1] on recovery of bacteria for meat samples and composite of all samples*

Incubation	Mean log count of organisms[2]				
35 C incubation:					
	C	A	D	B	E
Meat Products (n = 16)	5.5557	5.5807	5.5650	5.5648	5.4998
	A	C	B	D	E
All samples (n = 28)	5.3544	5.3450	5.3394	5.3044	5.2907
22 C incubation:					
	A	B	C	D	E
Meat products (n = 16)	5.9787	5.9646	5.9439	5.9395	5.8845
	A	B	D	C	E
All samples (n = 28)	5.6994	5.6825	5.6668	5.6568	5.6563
Pooled 35 and 22 C incubation:					
	A	C	B	D	E
Meat products (n = 16)	5.7797	5.7648	5.7647	5.7523	5.6922
	A	B	C	D	E
All samples (n = 28)	5.5269	5.5190	5.5009	5.4856	5.4735

[1]A = Butterfield's diluent, B = 0.5% peptone, C = 0.1% peptone, D = 0.85% NaCl, and E = Distilled H_2O

[2]Recoveries underscored by same line are not significantly different at the 0.05 level.

TABLE 3. *Summary of Friedman's ranking analysis of variance for the five diluents used[1]*

Incubation	Diluent				
	Butterfield's	0.5% Peptone	0.1% Peptone	0.85% NaCl	Distilled H_2O
35 C incubation:					
Meat samples (n = 16)	44	49	39	49	59
All samples (n = 28)	76	83	75	90	96
22 C incubation:					
Meat samples (n = 16)	41	44	44	49	62
All samples (n = 28)	69	81	82	90	94
Pooled 35 and 22 C incubation:					
Meat samples (n = 16)	40	48	43	50	59
All samples (n = 28)	71	85	79	91	94

[1]Highest Friedman ranking indicates lowest recovery level. No statistical differences are shown ($p > .05$).

samples analyzed were meat products. Analysis of data from incubation at 35 and 22 C revealed that Butterfield's diluent had the highest overall means and rankings with the exception of 0.1% peptone in the 35 C rankings, while distilled water had the lowest means and rankings. Analogous data from the meat samples showed similar results with 0.1% peptone having highest means

and rankings at 35 C. Such differences between results obtained at 35 and 22 C incubation could be attributed to each temperature supporting growth of bacterial populations comprised of organisms best adapted for growth at that particular temperature.

Although a trend is apparent in the effects of diluents upon recovery in this work, many samples showed considerable variability. Since neither the reason for the sensitivity of some bacteria to distilled water and saline, nor the apparent protection of some sensitive bacteria by peptones or buffered phosphates have ever been adequately explained, the complexity of sample-bacteria-diluent interaction still needs further investigation. Obvious differences in growth sustaining capabilities of these samples and their maintenance environment, and the nature and general viability of their bacterial populations as compared to other sample types could present a partial explanation. The pH of diluent was not seen to be a source of variability. The pH of all diluents was not affected by autoclaving and was relatively consistent. The average pH of diluents after autoclaving and cooling was 7.2 for Butterfield's diluent, 7.1 for 0.5% peptone, 7.2 for 0.1% peptone, 6.8 for 0.85% NaCl, and 7.3 for distilled water. In most instances, after mixing the food samples with the diluents, the pH was not significantly altered. However, with the dairy products examined, the pH of the homogenate was lowered to about pH 5.0 in all diluents.

Results obtained in this study indicate that Butterfield's diluent and perhaps 0.5% or 0.1% peptone may offer more consistent recoveries of bacteria from a variety of food samples than distilled water and 0.85% NaCl. However, Duncan's 5% level test and Friedman's ranking analysis of variance show no significant statistical difference between any diluents. Accordingly, there is no diluent that is ideally suited for every possible sample type, since each sample-dilution interaction seems to differ. More sampling within specific food groups would be desirable to obtain more definitive results.

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Effects of Added Copper on Concentrations of Volatile Materials Produced in Milk

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ABSTRACT

Analyzing the steam distillate from milk enabled measurement of increases in concentrations of n-hexanal, n-pentanal, propanal, acetaldehyde in raw, laboratory-pasteurized, and plant-pasteurized-homogenized milk exposed to 0 and 5 ppm added copper. Greatest changes were in the copper-treated laboratory-pasteurized milk and the least in Vacu-Therm pasteurized-homogenized milk. The 2-thiobarbituric acid values (TBA) in the exposed milk, laboratory pasteurized, .090; raw, .078; plant pasteurized, .056, reflected changes in volatile materials. Acetaldehyde concentrations increased about the same in whole pasteurized-homogenized milk as in skimmilk exposed to 1 or 2 ppm copper. The other carbonyl compounds increased consistently in whole milk, but little or none in skimmilk. Increases in concentration of the carbonyl compounds (other than acetaldehyde, acetone, and butanone) produced in pasteurized-homgenized milk exposed to 4 ppm copper for 44 h were reduced by adding 150 mg of ascorbic acid per liter of milk. However, the concentrations were greater in ascorbic acid-copper-treated milk than in milk not treated with copper. Adding the steam distillate from copper-oxidized milk produced the typical copper-oxidized flavor in good-flavored milk.

Oxidized flavors in milk and other dairy products have been studied extensively (*5, 12*). Though many carbonyl compounds have been isolated from oxidized dairy products (*12*), there is no clear agreement on which compund (or compounds) is responsible for the early off-flavor in oxidized fluid milk. Forss et al. (*6*) isolated and identified acetone, acetaldehyde, n-hexanal, crotonaldehyde, and series of C_4-C_{11} 2-enals and C_6-C_{11} 2,4-dienal from skimmilk oxidized with 5 ppm copper and 0.05 ml of 30% H_2O_2/ml and held 3 days. They found that 2-octenal, 2-nonenal, 2,4-heptadienal, and 2,4-nonadienal resembled the cardboard flavor in skimmilk at 10^{-7} —10^{-9} dilutions. Day and Lillard (*3*), working with oxidized milk fat, concluded that a wide spectrum of n-alkanals, alk-2-enals, alk-2,4-dienals, and alkan-2-ones was necessary to impart the oxidized flavor. Stark and Forss (*11*), who isolated and identified oct-1-en-3-one in oxidized milk fat, reported that it was responsible for the metallic flavor in dairy products. They also reported that when the compound was added to skimmilk, graders criticized the milk as "cardboard and oxidized." Day et al. (*4*) stated that although that compound produced a metallic flavor, it was not the oxidized flavor. Hammond and Seals (*7*) attempted to simulate the oxidized-flavor defect in milk by adding 1 or 10 ppb of oct-1-en-3-one and/or octanal to homogenized milk. They revealed that frequent criticisms among 53 judges of homogenized milk (in nine laboratories) to which the compounds had been added were: "typical oxidized," "metallic," and "cardboard flavor." Those same chemical compounds, however, also educed criticisms of "atypical" and of flavors other than oxidized, metallic, or cardboard much more frequently than did copper-induced-oxidized milk samples.

Interpreting early research on off-flavor compounds in oxidized products is difficult in part because then there were inadequate quantitative measurements. Much of the early research dealt with extensive oxidation or long storage periods of dried products, changes that may or may not apply directly to the "typical" flavor that occurs in oxidized fluid milk. The development of a simple method to measure minute (ppb) changes in volatile materials in milk (*2*) enabled me to study early changes in copper-induced oxidized flavor.

EXPERIMENTAL PROCEDURE

Measuring volatile materials in milk

Steam distillation and GLC analysis of the distillate were used to measure quantitatively the volatile materials in milk. Standard deviations of 10% of the mean value or less were obtained when four series of replicates of normal and 50 ppb spiked milk samples were analyzed for some volatile materials by this method (*2*). GLC peak components were identified by subtractive techniques (*1*). Aldehydes were distinguished from ketones by treating distillates with one drop of saturated potassium permanganate before the GLC procedure to oxidize and eliminate the aldehyde peaks. This is a modification of a procedure reported by Hoff and Feit (*8*).

Changes in concentrations of volatile materials in raw, laboratory-pasteurized, and commercial Vacu-Therm pasteurized-homogenized milk exposed to copper

A portion of raw milk collected at the surge tank of a DeLaval Vacu-Therm pasteurizer was laboratory pasteurized at 63 C for 30 min with gentle agitation in an Erlenmeyer flask. Shortly after the raw milk was collected, a sample of the Vacu-Therm pasteurized-homogenized milk (76.6 C/15 sec) was collected at the outlet pipe from the pasteurizer. Copper sulfate was added to aliquots of each of these milks to give a concentration of 5 ppm copper. A portion of each milk was untreated; it served as a control. All milk samples were stored in a refrigerator at 5 C for 23 h, then analyzed for volatile materials as described. The 2-thiobarbituric acid reaction values (TBA) were determined on the samples by King's method (*9*).

Volatile materials in commercial skimmilk and pasteurized-homogenized whole milk were compared after exposure to added copper

Samples of packaged skimmilk and whole, homogenized-pasteurized milk from the same milk supply (processed on the same day) were taken

from the University Dairy milk cooler. Aliquots from each carton were spiked with 0, 1, and 2 ppm copper as copper sulfate and stored at 5 C. These samples were analyzed for volatile materials, as described, at 16 and 45 h.

Effects of added ascorbic acid on volatile materials produced when copper was added to pasteurized-homogenized milk

Samples were from freshly pasteurized-homogenized, packaged milk from the University Dairy plant: control milk (no treatment); milk with 150 mg of ascorbic acid per liter; milk with 4 ppm added copper; and milk with 150 mg of ascorbic acid and 4 ppm added copper. These milks were analyzed for volatile materials at 20 and 44 h.

Organoleptic study of milk prepared by adding distillates from oxidized and control milk to good-quality milk

Pasteurized-homogenized milk exposed to 5 ppm copper for 45 h was distilled as described. The 5-ml distillates thus obtained were reconstituted into 45-ml aliquots of untreated homogenized milk and compared with the copper-oxidized milk and with the control milk diluted with distilled water (5 ml to 45 ml milk). Distillates from control milks also were blended with untreated milk (5 ml to 45 ml) and compared with all of the other samples. These milk samples were analyzed organoleptically by two experienced milk judges.

RESULTS AND DISCUSSION

Effects of some processing conditions on copper-induced production of volatile materials

Table 1 shows the concentration of volatile materials in raw, laboratory-pasteurized, and plant-pasteurized-homogenized milk exposed to 5 ppm copper for 23 h. The concentrations of each of the chemical compounds in the control milks did not differ appreciably (see raw, laboratory-pasteurized, or plant-pasteurized control milks, Table 1). n-Hexanal increased in concentration more than any other component in milk exposed to copper. Relatively large increases also were observed for n-pentanal, propanal, and acetaldehyde in milk exposed to copper. Concentrations of chemical compounds differed little in either copper-treated raw or laboratory-pasteurized milk. However, concentrations of all carbonyl compounds were lower in the copper-treated, plant-pasteurized sample, perhaps because the Vacu-Therm treatment of the milk, reduced the dissolved oxygen, before experimental treatment. Methyl sulfide did not change appreciably from exposure to copper. Several additional small peaks were not identified but these did not change with the treatments.

TBA values of milks exposed to different processing conditions and held 23 h without added copper were low, indicating no oxidation. The relatively lower TBA value for the plant-pastuerized sample than for the other samples exposed to copper generally paralleled the lower concentrations of volatile materials.

TABLE 1. *Changes in concentrations of some constituents of raw, laboratory-pasteurized, and plant pasteurized-homogenized milk exposed to 0 and 5 ppm copper for 23 h*

Treatment	Raw milk	Lab past.[a]	Plant past.[b]
	Acetaldehyde (ppb)		
Control	8.0	4.8	4.8
5 ppm Cu[c]	15.2	17.0	11.5
	Methyl sulfide (ppb)		
Control	10.2	7.1	7.8
5 ppm Cu	8.3	7.9	6.2
	Propanal (ppb)		
Control	2.7	2.4	2.4
5 ppm Cu	17.0	19.8	7.1
	Acetone (ppb)		
Control	385.5	306.5	295.5
5 ppm Cu	344.5	365.5	301.8
	Butanone (ppb)		
Control	60.0	47.8	47.1
5 ppm Cu	53.2	53.6	50.1
	n-Pentanal (ppb)		
Control	4.9	3.9	4.1
5 ppm Cu	96.0	114.7	48.5
	n-Hexanal (ppb)		
Control	4.9	3.9	4.1
5 ppm Cu	452.3	541.7	165.2
	TBA values		
Control	.019	.019	.013
5 ppm Cu	.078	.090	.056

[a]63 C for 30 min.
[b]Vacu-Therm, HTST pasteurized-homogenized.
[c]Held 23 h at 5 C.

Table 2 shows the changes in concentration of the volatile materials in commerical pasteurized skimmilk and pasteurized, whole, homogenized milk with 0, 1, and 2 ppm copper added. The consistency with which acetaldehyde increased with added copper in either skim or whole milk strongly indicated that the nonfat component of milk was the precursor of this compound. Increases in concentrations of the other carbonyl compounds were definite and consistent in the whole milk but slight or nonexistent in the skimmilk. From these data, I concur with the generally accepted view that lipids are precursors of these carbonyl compounds in copper-induced, oxidized milk. No precise pattern could be discerned for methyl sulfide in this study.

Volatile materials in skimmilk and whole-homogenized milk exposed to added copper

TABLE 2. *Changes in concentrations of some compounds in commercial skim and whole pasteurized-homogenized milk with 1 and 2 ppm copper added*

Exposure time (h)	Whole milk[a] added copper			Skimmilk[b] added copper		
	0	1 ppm	2 ppm	0	1 ppm	2 ppm
		Acetaldehyde (ppb)				
16	5.3	6.1	7.1	5.3	5.6	6.7
45	7.7	8.2	10.3	5.1	6.7	8.0
		Methyl sulfide (ppb)				
16	6.1	6.4	6.8	6.1	5.8	6.6
45	7.6	7.8	6.9	6.4	6.1	6.3
		Propanal (ppb)				
16	2.7	3.2	3.1	2.4	2.5	2.5
45	2.9	3.7	4.3	2.7	2.7	2.7
		Acetone (ppb)				
16	327.1	322.1	324.1	333.2	298.0	289.1
45	343.7	354.1	361.8	331.8	305.2	304.0
		Butanone (ppb)				
16	49.2	63.0	46.7	48.0	52.6	48.8
45	50.9	50.9	48.8	48.4	49.0	51.6
		n-Pentanal (ppb)				
16	6.0	9.6	8.9	-0-	-0-	-0-
45	11.0	19.7	25.4	-0-	-0-	-0-
		n-Hexanal (ppb)				
16	16.0	31.0	31.6	8.7	11.3	12.9
45	19.6	38.9	54.0	11.3	11.3	13.4

[a]Vacu-Therm, HTST pasteurized-homogenized.
[b]Commercial skimmilk with 0.14% fat and fortified with 1% NFDM solids.

TABLE 3. *Changes in concentrations of some volatile materials in pasteurized-homogenized milk with and without added copper and ascorbic acid*

Identified component[a]	Control		Ascorbic acid[b]		Added Cu[c]		Cu + ascorbic acid[d]	
	20 h	44 h	20 h	44 h	20 h	44 h	20 h	44 h
	(ppb)							
Acetaldehyde	7.5	7.7	7.4	9.0	10.3	15.4	8.0	15.1
Methyl sulfide	7.2	9.4	7.2	9.6	7.2	10.0	7.2	8.8
Propanal	2.9	3.1	2.8	3.1	3.8	5.4	3.0	3.6
Acetone	267.6	279.8	259.4	315.0	288.6	317.4	270.8	313.4
Butanone	65.8	65.6	62.5	74.4	70.0	74.7	64.4	73.4
n-Pentanal	21.1	21.1	17.5	21.8	29.0	44.9	25.4	31.9
n-Hexanal	32.1	31.6	32.6	35.8	61.2	104.4	54.5	68.5

[a]Identification based upon subtractive techniques and agreement of retention times.
[b]Added 150 mg of ascorbic acid per liter of milk.
[c]Added 4 ppm Cu as $CuSO_4$.
[d]Added 4 ppm Cu and 150 mg per liter of ascorbic acid.

Ascorbic acid and added copper as related to volatile materials produced in milk

The changes in concentration of the volatile materials in milk exposed to 4 ppm copper for 20 and 44 h with and without 150 mg ascorbic acid per liter are shown in Table 3. Obviously, ascorbic acid affected oxidative changes; the antioxidant effect, however, was relatively small. Though its effect was apparent at 20 h, ascorbic acid after 44 h seemingly inhibited the production of the lipid-related volatile compounds more than did the nonlipid-related acetaldehyde.

The carbonyl compounds that I measured have been identified in other studies of oxidized dairy products. Comparable increases in acetaldehyde in copper-exposed skimmilk and whole milk indicate that a nonfat component is its precursor. Propanal is the classical dismutation product of one of the linolenic acid hydroperoxides. The mode of n-pentanal formation in homogenized milk is not readily apparent, however, it appears to originate from the milk lipids. Skimmilk did not contain n-pentanal and none developed when it was exposed to copper. Hexanal is one of the dismutation products of the linoleic acid hydroperoxides and has been recovered consistently from oxidized fats. The quantitative measurements made in this study may be useful to those who may wish to further investigate organoleptic properties of oxidized milkfat. Similar measurements might be used to evaluate methods of controlling lipid oxidation.

Organoleptic properties of milk distillate-treated milks

Table 4 gives flavor scores and criticisms of coded milks prepared by mixing distillates from normal and copper-oxidized milk with untreated milk as compared with scores and criticisms of oxidized and control milks. Obviously, the distillates contain the oxidized flavor characteristics. The one judgment of "cooked" and "oxidized" for the control milk with distillate from the control milk may have reflected a carry-over judgment of a previous sample. Holding the refrigerated distillates 2 days eliminated the cooked flavor character.

A subsequent study in which the distillates of control and oxidized milks were added to good quality milk and compared with the control or oxidized milk confirmed findings shown in Table 4 (*10*). In that study, 3 to 5 panelists with 80 observations criticized all milks as oxidized when distillates from 1 or 2 ppm copper oxidized milk was added to good milk. When only 0.1 ppm copper-oxidized milk distillates was added to good quality milk, 79% of the samples (28 observations) were criticized as being oxidized.

TABLE 4. *Organoleptic properties of milks prepared by adding the distillates of control and oxidized milks (2) to good-quality milk*

Milk samples[a,b]	Judge 1		Judge 2	
	Score	Criticism	Score	Criticism
Control milk[c]	38.0	Sl. cooked	39.0	Cooked
Control milk[c]	38.0	Sl. cooked	38.5	Cooked
Distillate from Control milk[d]	37.5	Cooked	38.5	Cooked
Distillate from control milk[d]	37.0	Cooked	35.0	Cooked/oxid.
Cu oxidized milk[e]	33.0	Oxid.	35.0	Oxid.
Cu oxidized milk[e]	32.0	Oxid.	34.0	Oxid.
Distillate from Cu-oxidized milk[f]	33.5	Oxid.	33.0	Oxid.
Distillate from Cu-oxidized milk[f]	33.0	Oxid.	34.0	Oxid.

[a]Coded milk samples presented to judges in random order.
[b]When distillates were added to control milks, each sample was prepared from a new distillation.
[c]45 ml of pasteurized-homogenized milk + 5 ml of distilled water.
[d]45 ml of pasteurized-homogenized milk + 5 ml of distillate from the control milk.
[e]45 ml of Cu-oxidized pasteurized-homogenized milk + 5 ml of distilled water.
[f]45 ml of pasteurized-homogenized milk + 5 ml of distillate from Cu-oxidized milk.

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Determining Sanitary Status of Farm Milk Pipelines Using the Rinse-Filter Procedure

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ABSTRACT

Two farm milk pipelines with weigh jars and milk releasers were rinsed with water sterilized by membrane filtration. Samples of rinse water were collected and analyzed for viable bacteria and coliforms by the membrane filter method. Total aerobic counts averaged 7000/ft² of milk contact surface in a new milking system during the first month of operation. After 1 year of operation, when certain deficiencies of cleaning occurred, total counts averaged more than 500,000/ft². Upon correction of deficiencies, average counts dropped to 2000/ft². Counts of coliforms in clean systems averaged less than 1/ft². Gram negative bacteria constituted only 5% of the microflora in rinses from a system that had been in operation for 4 years; whereas, 67% of the colonies were micrococci and 22% were streptococci. Use of the filtration system for preparation of sterile water is discussed.

Proper cleaning and sanitizing are vital elements in production of high quality milk. Much time and effort are devoted to farm inspections and testing to help determine that milk is produced in a sanitary manner. Lately there has been considerable concern that the Standard Plate Count is unsatisfactory for indicating farm conditions. This can be especially true in those installations in which large quantities of milk pass over equipment so that contaminants are diluted to insignificant numbers.

One approach to control of equipment sanitation that is promising but infrequently employed is the rinse-filter technique. In 1918 Delephine, cited by Thomas (*12*), used rinses of sterile water for examining microbial content of milking pails in England, and numerous studies have since been reported (*3, 5, 6, 9-13*). Total aerobic microbial counts of 250,000/ft² were considered (*12*) indicative of satisfactory conditions for milk production. Druce and Thomas (*4*) provided an extensive review. Standard Methods (*1*) provides for circulating water which has been chlorinated and neutralized. The increase in bacterial content is determined by the membrane filter technique. Marshall and Appel (*8*) employed microfiltration to sterilize water which they used in rinse-filter studies in milk processing plants.

Of 18 milk pipeline systems studied by Jackson and Clegg (*7*), 50% were dominated by micrococci, 28% by streptococci, and 22% by gram negative rods. Other workers (*4, 12*) made similar observations except that Egdell and Widdas (*5*) found gram negative rods to be more dominant than streptococci.

The present paper reports on application of microfiltration to sterilize water, rinsing of farm pipeline milking equipment with the water, enumeration of microorganisms removed by the water, and identification of representative microorganisms.

MATERIALS AND METHODS

Rinse water was prepared by filtration of water from the farm supply through a wound, cotton string prefilter (type 12531, Gelman Instrument Co., Ann Arbor, Michigan) and then a cartridge type membrane filter (Millitube cartridge, Millipore Filter Corp., Bedford, Massachusetts) with a pore size of 0.45 μm (*8*). A sample was taken of the first water through the apparatus to test sterility. A delivery hose, which led from the filter apparatus, was connected to the appropriate inlet of the milking machine.

Before testing, the milking machine was cleaned by the usual cleaned-in-place procedure and sanitized with hypochlorite (200 mg/1) or acid type iodophor (25 mg/1).

Two DeLaval pipeline milking systems were studied. System 1 had four weigh jars attached to glass milk and air pipelines. System 2 had 12 weigh jars attached to stainless steel pipelines. Water was introduced into the milk pipeline and flowed by gravity to the weigh jars and the milk releaser jar where (in each) it was allowed to accumulate to a volume of 6.3 1. Before rinsing, 50 ml of 10% sodium thiosulfate were added asseptically to the jars to neutralize sanitizers. Samples were collected aseptically from the bottom of the weigh jars by removing the sanitized line and allowing solution to flow into a sterile plastic bag. A sanitized sampling port was used to collect samples from the milk releaser jar. In certain studies water was also introduced into the vacuum line and directly into the weigh jars.

Total aerobic bacteria counts and coliform counts were obtained using standard membrane filter techniques (*1*). Counts of thermoduric bacteria were obtained by filtering 300 ml of rinse solution through a membrane filter (0.45 μm). Filters with their microorganisms were placed individually in screwcapped tubes (25 × 150 mm) containing 10 ml sterile skim milk. Tubes were shaken to dislodge bacteria from the filters and were submerged in a water bath. After 30 min at 62.8 C, samples were cooled, and the thermoduric count was determined (*2*).

Representative colonies of each color and morphological type were picked from membrane filters onto Tryptic soy agar slants. Colony characteristics and percentages of each type were recorded. Gram-stained smears of each culture were examined and tests were performed on each group as follows: (*a*) gram negative rods-oxidase production and lactose fermentation; (*b*) gram positive rods-catalase production and reaction in litmus milk; (*c*) gram positive cocci-catalase production, colony morphology, and hemolytic pattern on blood agar, and colony characteristics on Baird-Parker agar. Cultures which failed to fall into a distinct group were placed in the miscellaneous category.

RESULTS

Experiments with system 1

This milking system was installed in 1969 and had been cleaned-in-place routinely after each milking. Rubber parts were replaced irregularly when they appeared to be damaged or difficult to clean. At the time sampling was started, rubber parts showed slight evidence of deterioration and the maximum attainable temperature of the cleaning solution was 55 C. During recirculation, the temperature dropped to 43 C. Under these conditions a series of three rinse tests disclosed a log average of 200,000 bacteria per ft² of milk contact surface (Table 1). Rinse solutions for these tests were passed through the milk pipeline into the weigh jars and the milk releaser jar. Temperature of the cleaning solution was then lowered to 46 C (it dropped to 38 C during cleaning), and another series of three rinse tests was done. No effect was observed on the numbers of bacteria recovered (Table 1).

TABLE 1. *Bacteria counts from rinse-filter tests of pipeline system 1 when cleaned using two different temperature ranges*

Temperature range (C)	Total count ($\times 10^4$)[b]/ft² (Sampling point[a])					
	1	2	3	4	5	Log Avg.
55-43	18	40	29	26	6	20
46-38	5	34	20	40	7	16

[a]1-weigh jar 1, 2-weigh jar 2, 3-weigh jar 3, 4-weigh jar 4, 5-releaser jar rinsed through milk line.
[b]Log average of three rinse tests.

Two commercial chlorinated detergents were compared for cleaning effectiveness at concentrations recommended by the manufacturers. Available chlorine concentrations in solutions of cleaners 1 and 2 were 11 and 50 mg/1, respectively. Total bacterial counts were substantially lower throughout the system when cleaner 2 was used (Table 2).

TABLE 2. *Bacteria counts from rinse-filter tests of pipeline system 1 cleaned with two different detergents*

Detergent	Total count ($\times 10^4$)/ft² (Sampling point[a])						
	1	2	3	4	5	6	Log Avg.
1[bc]	21	200	37	84	1.0	11	23
2[de]	.2	81	24	7	0.3	2	5

[a]1-weigh jar 1, 2-weigh jar 2, 3-weigh jar 3,4-weigh jar 4,5-releaser jar rinsed through vacuum line, 6-releaser jar rinsed through milk line.
[b]11 ppm available chlorine.
[c]Log average of 3 rinses.
[d]50 ppm available chlorine.
[e]Log average of 4 rinses.

Coliform densities were measured in 10-ml aliquots of rinse solutions. Largest concentrations were observed in samples which had been flushed through the vacuum line into the releaser jar. Eighty-six percent of these samples contained coliforms (in numbers up to about 10/ml), compared with 56% positive samples from rinsings of the milk line into the releaser jar. Only 20% of the 10-ml samples from the weigh jars produced coliform colonies despite the presence of several hundred bacteria/ml in most rinse solutions.

The percentage of the total bacteria which survived laboratory pasteurization (in seven rinse trials and six sampling locations) averaged 1.9 (logarithmic average) and ranged from 0.2 for weigh jar number 4 to 10 for weigh jar number 3.

Among all the isolates micrococci were the most numerous, representing 67% of the colonies on filters from 11 rinses and six sampling locations per rinse. Nearly 22% of the colonies were streptococci. Pseudomonads and other gram negative rods constituted only 5% of the colonies, whereas gram positive rods constituted 6%. About 1% of the colonies were yeasts.

Water was introduced directly into weigh jar number 1 whereas it passed through the milk pipeline and connecting hoses into the other three weigh jars. Total counts in jar 1 were only about 13% of those in the other jars which suggested that the rubber connecting hoses were heavily contaminated.

Experiments with system 2

This automated, cleaned-in-place milking system (Fig. 1) consisted of 12 weigh jars (six per side), and samples were taken from every-other jar after introduction of sterile rinse water into the milk pipeline at one end. Rinse filter tests were done three times during the first

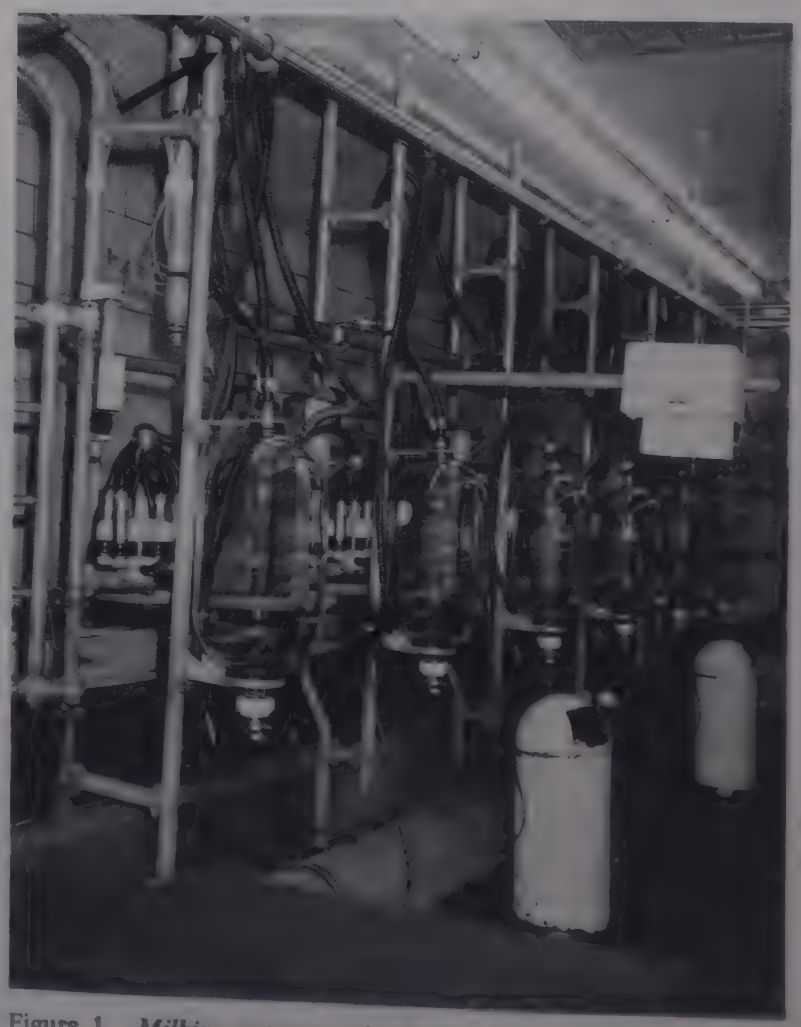

Figure 1. ***Milking system number 2. Rinse water was introduced into the milk pipeline at upper left (arrow) and flowed down through the connecting rubber hoses into every-other weigh jar. Another set of 6 weigh jars was located on the right, and 3 of them were rinsed.***

through fourth weeks after installation and then four times during a 4-week period 1 year later. Temperature of the cleaning solution during the first study was 71 C, and it dropped to 60 C during recirculation. However 1 year later the initial temperature was only 55 to 60 C and the quantity of water dispensed by the automated system was about 5 gal. less than optimal.

Counts from the initial series (new system) averaged 7000/ft². One year later and under conditions described above, the series of four rinse tests of the same line and six jars produced counts which averaged over 500,000/ft². However, when temperature and quantity of the cleaning solution were raised to the original levels and the system was thoroughly cleaned, bacteria counts dropped to an average of 2000/ft². The cleaning process included manual cleaning of some parts and tightening of loose gaskets.

Coliform bacteria were present in only 3 of 18 rinse samples taken during the first 4 weeks of operation. Before adjustment of the CIP system 1 year later, 19 of 24 samples contained coliforms. After adjustment, six rinse samples (100 ml each) contained no coliforms.

DISCUSSION

These observations demonstrated the usefulness of the rinse-filter technique for determining the sanitary condition of farm milk pipeline systems. Tests made on the new milking system demonstrated that numbers of recoverable aerobic bacteria can be as low as a few thousand per square foot of milk contact surface in piping, tubing, and weigh jars. Counts of coliforms in clean systems should be less than 1/ft² of surface. High counts, which often exceeded 200,000/ft² of milk contact surface, were associated with deteriorated rubber parts, loose gaskets, low temperatures of cleaning, and insufficient cleaning solution. Obviously, several other factors may have affected counts.

Quantities of rinse solution which should be filtered to obtain countable numbers depend on the amount of surface area rinsed and the number of bacteria thereon. However, our tests employed 1 to 100 ml for total aerobic counts and 10 to 200 ml for coliform counts.

An apparatus such as we used to sterilize rinse water can be constructed for less than $300. With precautions against introduction of rusty or turbid water the filters remain serviceable through many uses. The cotton string filter can be rejuvenated by soaking in a 20% solution of hot acid cleaner. The same treatment often clears the pores of the membrane filter.

The greatest problem facing the sanitarian in field use is keeping the apparatus sterile on the downstream side of the filter. Joints must be kept tight so no leaks develop. The end of the hose which is connected to the milker must be considered contaminated once it is disconnected, and the end must be decontaminated. Furthermore, water in this hose must not be allowed to flow back into the apparatus (as may happen if the configuration of hoses allows back-siphonage). The procedure we recommend is as follows: (*a*) shut off water to filters, (*b*) disconnect delivery hose from milkline, (*c*) submerge delivery hose in sanitizer solution (200 mg/l hypochlorite or 25 mg/l acid type iodophor), (*d*) disconnect water supply, and (*e*) cover end of delivery hose with a sterile plastic bag. A valve may be located in the water line ahead of the membrane filter. Closing of this valve will prevent flow of water within the apparatus after it is disconnected. Thus chances of contamination are decreased.

In some installations it is difficult to introduce sodium thiosulfate to inactivate residual sanitizer. A satisfactory alternative is the addition of 1 ml of 10% sodium thiosulfate to the sample (We suggest collecting about 250 ml in an 18-oz. plastic sample bag). The low concentration of sanitizer present should cause negligible reductions in count during the few minutes required to take samples. (We were able to complete the entire operation in less than 30 min.)

These data are insufficient to conclusively indicate what numbers of bacteria should be permissible in milking systems. However, they indicate that extremely low counts are attainable, and they strongly suggest that surfaces are unsanitary when numbers exceed 200,000/ft² of milk contact surface. (Contact surface area is as follows for several commonly used components of pipeline milkers: (*a*) milk hose-28 in²/ft, (*b*) 2-inch milk line-75 inch²/ft, and (*c*) glass weigh jar-1430 inch².)

Gram negative bacteria were a small proportion of the microflora rinsed from system 1 which was in only mediocre condition and was being cleaned with solutions at relatively low temperature. Assuming that system 1 was uniformly contaminated throughout and that the rinse water removed all the organisms (extremely unlikely), about 1,000,000 gram negative bacteria would have been contributed to milk when gram negatives made up 5% of 200,000 microorganisms per ft². This would provide about one gram negative bacterium/ml of milk in a 1000-gal tank (assuming ¼ capacity filled per milking). Whether this number would be significant depends on the type of organism and time and conditions of holding the milk.

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Planning for Food Protection During the Bicentennial Celebration in the Nation's Capital

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ABSTRACT

The potential for foodborne disease outbreaks is increased during mass feeding activities and periods in which food services are overtaxed; this is a major concern of health agencies throughout the United States as they prepare for the Bicentennial celebration. As the District of Columbia government has had some experience in planning food protection services for intense activities generated by large influxes of people, it has developed a comprehensive food protection program in preparation for the Bicentennial activities. Included in the District government's plans is the implementation of a "self-inspection" program to be conducted by trained and certified food service workers in selected food service establishments throughout the city. While the District government cannot guarantee that no food borne diseases will occur during the Bicentennial celebration, the food protection program will maximize its efforts to ensure that consumers are protected against food-borne health hazards.

The history of community health problems is replete with examples of foodborne disease outbreaks which have occurred because of deficiencies in food service hygiene and sanitation, especially in mass feeding activities and during periods in which food service facilities were overtaxed. It is during these peak periods of food service that large quantitites of foods are continually emerging from the preparation and service levels of the food distribution system. As such, a microbiological hazard, including contamination or infection by bacteria, viruses, or proteozoa, should it develop at some point, could impact on the health of a large number of consumers.

Consequently, the Government of the District of Columbia is concerned about microbiological hazards in foods not only during the forthcoming Bicentennial celebration, but at all times, and has developed programs and services designed to remove the potential for food contamination and infection in public eating places of the Nation's Capital.

This program, like regulatory food protection services in most urban areas, covers all eating and drinking establishments where foods or beverages are served or otherwise provided for the public, as well as in those kitchens and commissaries and similar food-preparation establishments which are used for the final preparation of food services for consumers in other locations. We recognize that it is not sufficient to apply food protection standards to food service establishments only. The source of food, food products and ingredients, as well as transportation methods of all foods utilized in the community, are subjected to control.

Worthy of special note are mobile food units which are being utilized in rapidly expanding numbers near the White House, the Lincoln Memorial, the Washington Monument, and other points of major historical interest in Washington. Such units must also adhere to all applicable food sanitation standards and practices designed to prevent foodborne illness.

Our experience also indicates that too few food protection services take an active part in the supervision of food-service operations in churches and institutions affiliated with religious organizations, and private, social, or political clubs. All of the food service operations in the District of Columbia are now under the regulatory control of the D.C. Environmental Health Administration and are routinely monitored.

The number of persons served food by such institutions is high and because food-sanitation principles are often not observed, the hazards in terms of potential foodborne illness are significantly greater than in food service by establishments under regulatory agency supervision.

FOOD PROTECTION ELEMENTS

Against that background it is well at this point to reemphasize essential elements of a food protection program and discuss these with reference to the District of Columbia's current regulatory program and plans for food protection during the Bicentennial celebration.

Fortunately, in the District we have had some experience in planning food protection services for intense activities generated by large crowds of people such as those anticipated during the Bicentennial celebration. Every 4 years the Presidential Inauguration attracts thousands of additional visitors to the Nation's Capital, and during that period the quantity of food distributed and/or served increases almost threefold, requiring stepped up surveillance by District regulatory agencies.

One of the first essential elements of a food protection

program is the establishment of standards—the requirements which must be satisfied for all aspects of sanitary food distribution. Early in 1974 the District of Columbia City Council updated by amendments the D.C. General Food Regulations in recognition of the newer developments in food protection.

These regulations, which had consumer and industry input to their development, provide a firm basis for the implementation of a modern and effective food protection program.

One highly significant amendment to the regulations is the requirement that all food service managers demonstrate their knowledge and understanding of the basic principles of food sanitation and of the prevention of foodborne illness.

As the first municipality to require the certification of food service personnel, the District of Columbia recognized that the education of food service workers and managers is the foundation of good food sanitation practices. We also recognize that the successful public health sanitarian in this field is the one who is successful in his educational program and not one who is most eager in his desire to prosecute.

To date 1,740 of approximately 3,000 food service managers have been trained and certified. By October 1975, 3,000 food service managers employed in District food service facilities will be certified.

In effect, these trained and certified food service workers will serve as auxillary in-house "inspectors" of the establishment in which each of them will be employed, and they will add a new dimension to enlightened food service management.

We hope to rely heavily on these food service managers for the day to day management of in-house food sanitation programs during the Bicentennial celebration.

MANPOWER AND RESOURCES

While comprehensive codes and ordinances are desirable they are no substitute for well-qualified, well-trained, and competent personnel to carry out necessary monitoring and surveillance.

We readily admit that the recruitment and retention of qualified personnel are difficult aspects of program administration at all levels of regulatory work. Part of the problem evolves from the fact that the quality of food protection programs is often not a sufficiently challenging opportunity for professional career development.

Potential candidates for employment in food protection services soon lose interest in a program which limits opportunity for promotion. The opportunities to advance, to conduct research, to participate in shaping programs, and to resolve difficult problems, are necessary components of career development and job satisfaction.

Moreover, many non-professionals have "drifted" into this field and have attempted to lower the personnel standards by suggesting that persons with less than an associate or baccalaureate degree in the physical or biological sciences or similar training or experience can carry out the intricate dimensions of food protection.

The suggestion may have been justifiable decades ago when the food industry was not as complex and as highly developed as it is today, or when ex-meat cutters or ex-bartenders or ex-military cooks without formal training in the physical or biological sciences were considered highly qualified for "food inspection." But in today's food industry, which is intimately involved in the rapidly changing attitudes and value systems that are reshaping the fundamental underpinning of our society, it is essential that public health workers in food protection services have educational and experienced backgrounds which qualify them to engage in the technical and professional aspects of food hygiene.

Since new personnel having the necessary academic preparation in the basic sciences often lack experience in food hygiene and sanitation, we have developed an upward mobility career ladder, an on-going training and orientation program, including seminars, meetings, and formal lectures designed to develop a competent food protection staff.

This in-service program will continue during the Bicentennial celebration and at the same time we will increase the size of our current staff by seven public health sanitarians, an applied microbiologist, and a health educator.

These new positions will be established and recruited with funds provided by the U.S. Food and Drug Administration (FDA) and they will complement our current staff of public health specialists.

We are encouraged by the outstanding support and cooperation we have received from the FDA, through our contractual agreements. As those of you who have worked with the Agency already know, FDA is no stranger to cooperative undertakings with state and local agencies and with private industry. It will continue to work with us in assuring uniformly safe foods for the consumer during the Bicentennial and long after the celebration has ended.

PLANNING AND ORGANIZATION

However, a technical staff and supporting services cannot be effective without being properly organized and directed. To this end we have developed a comprehensive plan which spells out specific goals and objectives and identifies milestones and strategies for deploying resources for maximizing our food protection services during the Bicentennial. For example, one of our major objectives will be to evaluate the effectiveness of the training and certification program for food service managers. Linked to this objective is our desire to develop and evaluate a self-inspection program for selected food establishments. It is here that management will be encouraged to accept major responsibility for the hour to hour food sanitation practices in their establishments. This logically assumes that management

realizes that the consumer desires and deserves high quality food sanitation.

In the areas of highest tourist impact, food service facilities will be evaluated monthly instead of the current quarterly schedule. The primary rationale for this increased surveillance is that food protection programs are preventive programs which include the provision for readily-available consultation to the food industry in determining the nature and extent of sanitation hazards, as well as defining corrective actions to be taken during intense periods of food service, when large numbers of visitors overtax the food management resources.

As a part of this effort we will continue to provide assistance in meal planning which includes menu review and analysis. This service has been a very productive approach in the past. It simply requires that our food protection staff thoroughly review the menu of each large official function (state dinners, receptions, banquets) at least 10 days in advance of the meal.

Our review pays special attention to foods consisting, "in whole or in part, of milk, milk products, eggs, meats, poultry, fish, shellfish, or other ingredients capable of supporting rapid and progressive growth of pathogenic microorganisms." In this way we can identify early potentially hazardous foods and ensure that they are properly managed under optimum conditions.

This service also provides an opportunity to pre-schedule whatever sample collection may be necessary for preventive microbiological analysis of these foods before they are consumed.

Unquestionably, this is an important service because the microbiological safety of foods available to the consumers is entirely dependent on management's awareness of microbiological hazards, the integrity of its food sanitation program, and the effectiveness of applicable government regulatory surveillance programs.

COMMUNICATIONS AND INFORMATION

Since communication and information are essential elements in any effective program or service we will install a "24-h hot line" to receive and cause prompt investigation of all complaints about food service hygiene and sanitation. This system will also provide a means of providing consultation and advice on a whole spectrum of food protection issues which could arise during the Bicentennial celebration.

Complementing this activity will be other consumer-oriented information channels. *The Consumer Information Bulletin,* currently a weekly compilation of District public food service facilities whose licenses have been suspended because of substandard food service management, will be published daily during the Bicentennial. Not only will it include restaurant closures, the so-called "No-No List," but the *Bulletin* will contain items of one-time or short-term interest to the consumer as well as the food industry.

Additional efforts to keep the consumer informed will include regular consumer orientation sessions. These will be conducted in conjunction with the D.C. Office of Consumer Affairs' continuing education services for all consumer groups.

All too often misinformation has resulted in an erosion of consumer confidence, and a lack of community support for food sanitation programs. Our thrust in public awareness has been, and will continue to be, to maintain a high level of consumer trust and at the same time ensure that consumers are well enough informed so that their demands are in keeping with what can be supplied from a scientific, technical, or even practical standpoint.

Clearly, the principles of public relations should without a doubt be constantly practiced in the course of executing a food-sanitation program. We are dealing with people and every legitimate practice and technique must be employed to interpret the program's needs and objectives and to motivate people to cooperate.

In this direction the mass media of communication have been among our most important allies, and they have been helpful in selling the need, importance and benefits of a comprehensive and effective food protection program. The cooperation of the two major Washington newspapers and the four television stations have been beyond reproach.

These then, in fairly broad outline, are some of the basic considerations which are shaping our plans for minimizing foodborne illness during the Bicentennial celebration in the Nation's Capital. While we cannot guarantee that no foodborne diseases will occur, every effort is being and will continue to be made to ensure that: (*a*) food is protected against dangerous microbial contamination; (*b*) food is sound, clean, and free from adulteration, and otherwise suitable for human consumption; and (*c*) consumer expectations are met; this dictates that food, in addition to being safe, is appealing and served in a pleasant surrounding.

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The Federal Food Service Program

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ABSTRACT

The Division of Food Service, U.S. Food and Drug Administration, is concerned with food safety when food is offered to consumers for consumption. This includes an estimated 600,000 institutional and commercial food service establishments, 300,000 retail stores, and vending machine sites numbering in the hundreds of thousands. Plans to accomplish this mission include achievement of uniform regulations and uniform enforcement procedures nationwide based on FDA standards, providing and/or supporting training and certification programs for regulatory officials and industry, developing and supporting self-inspection/quality assurance programs, evaluation of State programs, technical assistance to State regulatory agencies, and development of a model data processing system to aid program administration at the State level.

The fourth largest business in the United States is the food service industry. It consists of some 600,000-plus food service establishments that do an estimated gross of 62 billion dollars a year. Such establishments employ over four million persons. Add to this some 300,000 retail food markets and thousands of vending machine locations and it is plain to see that our work is cut out for us.

The Division of Food Service of the U.S. Food and Drug Administration is concerned with food safety wherever food is offered to consumers for consumption. More than 30% of meals consumed in the United States are prepared outside the home. Actual incidence of illness attributable to food is unkown, but certainly is many times greater than the 12,500 cses reported in 1973. Although our technology has increased over the past 40 years, we still seem to be unable to prevent food-borne outbreaks. We know what should be done, but apparently we haven't been able to get the message across to the people who can reduce the risk of food-borne illness—the food service operator and his employees.

With this in mind, The Food and Drug Administration is embarking on a new course. In the following discussion I shall outline some of those ideas.

FOOD SERVICE CODE

Many readers are familiar with the recommended 1962 Public Health Service Food Service Sanitation Ordinance and Code. On October 1, 1974, a proposed regulation to revise that code was published in the *Federal Register* for comment. During the 90-day comment period, plus an additional 30-day extension, 353 separate letters and memoranda were received containing approximately 3,300 comments. Each comment was considered and covered in our prepared Preamble to the final order which will soon be published in the *Federal Register*. The Preamble alone, giving rationale for accepting or rejecting the comments, came to over 230 typed, double-spaced pages. This should point out the thoroughness, and the interest in obtaining a truly uniform, workable regulation.

While the regulation will be used by the Food and Drug Administration for direct inspection in the interstate travel sanitation (interstate carrier) program and in our contract work in inspection of food service facilities in federal buildings controlled by government service administration, it is obvious that the Food and Drug Administration could not inspect nor regulate more than an insignificant part of the 600,000 food service establishments. We shall continue to rely on State and local regulatory agencies to carry the burden of these establishments. To assist in this program, there will be a Model Food Service Ordinance, identical in technical requirements, that will be available and promoted for State and local adoption.

Earlier I mentioned 300,000 retail food markets (grocery stores, bakeries, etc.). Because of a number of requests from State and local regulatory agencies, and incidentally, industry, we are presently preparing a model ordinance. This will be available for comment, we hope, by the end of this calendar year (1975). Also, by July 1976, we hope to publish, for comment, a revision to the Recommended 1965 Vending Machine Ordinance and Code.

What are the advantages of uniform regulations and uniform enforcement? Many companies operate food service establishments in several State and local jurisdictions. As a matter of fact, the 50 largest limited menu, fast food franchise companies have an annual gross of nearly 7.5 billion dollars of the 62 billion total. The top three franchise companies, McDonald's, Kentucky Fried Chicken, and Burger King, having over 7,600 food service establishments, account for over 40%

of the 7.5 billion dollars worth of business. It is not logical to require these companies to vary their sanitation practices for every franchise in a different geographical location. The national standard is a consensus standard—it will adequately protect the consumer. Therefore, it should be applicable throughout the United States. If the operator knows what is required of him—if the standard is practical and he understands it—he is more likely to comply.

I must point out that merely revising an ordinance does not solve all the problems. The true measure is found in applying that ordinance—education and firm, equitable enforcement.

ENFORCEMENT

In speaking of enforcement, I want to touch on a growing trend in the United States. In several areas regulatory agencies have gone to the news media to publicize violations in food service establishments by naming those failing to meet minimum sanitation requirements.

While this may be effective for a period of time, I wonder what will happen when public interest wanes. It seems to me that in these cases the regulatory agency is not doing its job, but is relying on the public to do its work. Why hasn't action been taken to bring about compliance rather than merely stating "this food service establishment is unsafe to eat in?" Why hasn't the regulatory agency, through its compliance procedures, required the operation to cease until it met minimum sanitation standards? Am I, the consumer, required to read a local newspaper to note the places I should not go for a meal rather than rely on the regulatory agency to protect me by making certain these poor operations do not serve food? After legal action is taken or the establishment closed, I would have no objection to publication of this fact.

ADMINISTRATIVE SURVEY

In another facet to try to bring about uniformity, the Food and Drug Administration will begin doing an administrative survey of all the States during this fiscal year. We should know more about what assistance the State needs so we can plan our program on the federal level to find answers to these needs. We shall also be encouraging the State to do more evaluation of local food service sanitation programs. To assist in this, we have rewritten the *Procedures for Evaluation of Food Service Programs.* This procedure will soon be available to the States. Through this evaluation procedure, we can expect more interest in the area of food service and a higher priority given to this area of work. The results will better pin-point the specific problems; then progress can be made toward solving these deficiencies.

STANDARDIZATION

If we want the procedures to work, we must standardize the persons who will be doing the evaluations. There are presently three persons in the Division of Food Service who standardize the Senior Regional Food Specialists in each of our 10 regional offices. These specialists in turn standardize additional Food and Drug Food Service personnel in the regions. The regional standardized staff then standardize and certify State personnel who will be evaluating local programs and training local food service regulatory personnel. As a side light, there was an interest expressed by Canadian officials in our standardizing program. As you are well aware, there are aircraft flights daily between our two countries. In the United States, the Interstate Travel Sanitation Branch has the direct jurisdiction for any food being placed on interstate carriers to serve the passengers. Through an agreement with the Canadian Department of Health and Welfare, Gary Kaar, Environmental Health Officer, was standardized by one of our staff last winter. He will now be making inspections of catering operations at some of the aircraft terminals that place food on planes flying to the United States. I'm sure he can confirm that the standardization program is not a "window-dressing" function, but requires lots of "book work" plus field demonstration that requires competency.

TRAINING

In spite of our efforts over the past few years, sanitation problems still exist in many food service establishments. Probably tens of thousands of food handlers' courses have been held over the past 30 years, usually sponsored and taught by regulatory officials. For the most part, these courses were not effective, nor did they come close to training the four million food service employees. From my own experience at the State level the owner or operator seldom if ever attended such sessions.

In an effort to improve the courses, and to get to the persons actually responsible for the food service operations, i.e. those that set the "tone" as to how their establishments would operate, the Food and Drug Administration is trying a new approach with the help of the States.

During the past 2 years, the Food and Drug Administration contracted with the Ohio Department of Health to develop an Owner/Operator/Manager Food Service Training Program. Ohio's course curriculum included discussions in biology, bacteriology, the causes and spread of diseases through food, food-service regulations, insect and rodent control, environmental health principles, food service plans and equipment review, personnel training, and industry self-inspection. Those who have attended the course and successfully passed the examination (approximately 750) have been listed by Ohio as certified food service managers. For the most part, these managers have been enthusiastic about the course. The "proof of the pudding" is now being checked. Has the course resulted in the manager upgrading his sanitation practices? This aspect is now being checked through comparison of establishment inspections made after attendance with those inpections

done before the manager attended the course.

As a follow-up to test the validity of Ohio's experience, the Food and Drug Administration awarded contracts to the States of Colorado and Virginia to use the same basic curricula in training programs within those States. Colorado added some additional material such as the economics of food service operations, including accounting procedures, insurance and safety, to augment the sanitation content. Final reports from these States on the contracts have been received but at this time have not been thoroughly evaluated. When the evaluation is completed, we shall prepare a model food service certification program that we hope all States will adopt.

While such a program will be voluntary at first, eventually we believe it should be mandatory. Persons who wish to operate a food service establishment should demonstrate they know and understand the principles of food service sanitation and protection of the public before they are permitted to operate such an establishment. As it now stands, anyone with sufficient capital and the inclination can enter the business even if he knows nothing about protecting the public from foodborne illness.

Even with the start we have made there are still many problems associated with the training of owners/operators/managers. Right now there are a potential one-half to one million persons to be trained. Obviously, the regulatory agencies are not able, with present limitations of staff and funds, to do all the training needed. We shall have to rely on junior colleges, vocational schools, and other institutions to participate, on a cooperative basis with the regulatory authority, in presenting classes to the potential attendees. The managers, after certification, will be expected to teach their own employees. In essence, we are saying "train the trainer." Contracts have recently been awarded to three regulatory agencies to cooperate with schools in utilizing academic staff in training the operators.

We are very happy to see industry accepting the challenge. The National Restaurant Association has published several sanitation brochures, prepared excellent audio-visual training materials, and has challenged State restaurant associations to improve their sanitation image. They have participated with the National Institute of the Food Service Industry (NIFI) in providing training materials and textbooks to further training. The sanitation self-inspection manual developed by the National Restaurant Association, and the new textbook, *Applied Food Service Sanitation*, developed by the National Institute of the Food Service Industry, are excellent study materials for food service personnel.

There is a word of caution, however. While most training is good and should be encouraged, I believe we should proceed slowly on official certification until such time as a uniform program is available. By uniform, I mean in minimum subject matter, not in method of presentation. As you know, food service workers, including managers, are mobile. We do not want a wide variety of programs, varying in length of course, type of subject matter, or depth of material presented, dependent solely on the location in which the owner/operator/manager attends such a program. What we would like to see is a model program, conducted under state auspices, covering certain designated subject areas so that reciprocity can be given between States.

SELF-INSPECTION

The next logical step after certification of food service managers is to establish a program of self-inspection or quality assurance. I would expect that this might be a tougher concept to sell to regulatory officials than to the food service operators, although we have been told by one State, Florida, that they are interested in trying this concept. We do, however, hear regulatory officials saying, "It's been tried before—It didn't work then and it won't work now." Admittedly, it has been tried before, but not too successfully. Why? We didn't do the first part. We didn't train the operator as to what he should be looking for. We believe self inspection can work if we proceed in the manner we have begun.

We have just recently received final reports on background data on fast food, limited menu food service establishments. These reports were the results of contracts with the States of Florida, South Carolina, and West Virginia. The contracts called for evaluation of such establishments to try to establish the critical sanitation points for a particular type of operation. Concurrently, the Food and Drug Administration, Division of Food Service, is working with the headquarters franchising office of two of these national companies. As you may know, most of the fast food, limited menu franchises prepare rather complete sanitation manuals for their particular operations, and require potential franchisees to attend company-sponsored training sessions. We have reviewed these sanitation manuals and have offered comments to the companies when appropriate. We believe if we can point out particular critical sanitation points within a company's operation, the information can be stressed in the training sessions sponsored by the company as well as written in their sanitation manuals.

We also want to work with the headquarters supervisors or auditors or whoever may be checking on company or franchise operations. If we teach them the specifics they need to watch for, they in turn can emphasize this to the franchisee and can train the operator in making self inspection.

The State or local regulatory official, by reviewing the food service establishment with the operator, and through joint inspection with him, can be convinced that the operator is capable of doing self inspection and will agree to file his reports with the regulatory agency as to the defects he found and the corrective measures taken. The regulatory official can monitor these reports and actually inspect the establishment once or twice a year to

determine the validity of the self inspection report. In this manner the regulatory agency could decrease the number of official inspections to the minimum required by law in cooperating, generally non-problem establishments, and increase the number of inspections in problem establishments.

Two questions may come to your mind. Why did we start with fast food, limited menu franchise units, and will all establishments in a jurisdiction be under self inspection?

We started with fast food, limited menu, franchise units for two reasons. One, they do have a limited menu, are usually uniform in design and operation, and usually provide training to their franchisees. Therefore, the basics are already available to start a program. Second, recent statistics indicate that these types of establishments are responsible for serving possibly the greatest number of consumers a day.

Now, will all establishments be under self inspection? The answer is "No." Some operators won't want to participate, and others, particularly the problem operator, could not be trusted to file an adequate, complete, honest inspection report.

For a program such as this to be effective, there must be adequate training of both the operator and the regulatory official, and mutual respect and trust between them. I believe this is possible if we really want to try something "new" and if we want the program to succeed.

RECORDS

In our evaluation of State programs, one of the deficiencies we most often note is in the records keeping system. During the past year we have, by contract with a private industry firm, developed a model automatic data processing system to be used by States and local regulatory agencies as an aid to administration of these programs. This system, called Sanitation Programs Information Formulator (SPIF), will be tested this fiscal year in a few cooperating States.

Very basically the system was developed to be introduced with the recommended 1975 Food Service Sanitation Ordinance as a tool that States could use to collect, store, and retrieve inspection information on food service establishments. Through the use of the computers it would be possible to make analyses of many factors of the program, determine problem areas, aid in measuring program effectiveness or program deficiencies, and point out areas in which shifts in program priorities should be made. Another aspect is the possibility of employing SPIF as a cost-effective device to aid in evaluation of effectiveness of an innovative sanitarian training program. During the development of SPIF, about 20 State program administrators acted as an advisory group, and for the most part, they indicated the system had a great potential.

TEMPERATURE MEASUREMENT

I would like to mention one more modest contract that we have entered into with the National Sanitation Foundation. For years the field sanitarian has been testing dishwashing machine temperatures with a maximum registering, mercury-filled thermometer. We have recognized the problem of trying to arrive at a temperature measured at dish level that would assure us that the dish is adequately sanitized. The National Sanitation Foundation will be trying to establish and validate practical methods to field-test these temperatures. This may involve correlations of heat unit equivalent accumulation with temperatures measured by maximum registering, mercury-filled thermometers, and heat sensitive tapes, and correlating these readings with the thermometer installed on the machine that measures temperatures at the rinse manifold. In other words, we want to determine the correlation between time, temperature, and final rinse water volumes needed to assure a sanitized dish.

It is evident that we are trying to break out of the history of the past—to become more flexible and improve our methods of dealing with new technology. The programs we have been trying over the past 40 years have not kept up with the changing technology and the way the business community operates today. We must continue to strive for increased understanding and innovation if we are to get the task accomplished.

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Experimental Anisakiasis: Cultivation and Temperature Tolerance Determinations[1]

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ABSTRACT

All stages of the anisakine life cycle—egg, larva, and adult— have been used to initiate cultures. Eggs develop to hatching larvae at different temperatures depending on the species or strain. Newly hatched larvae of *Phocanema decipiens* and *Contracaecum osculatum* grow to an average 31.1 mm in 52 weeks and 6.5 mm in 32 weeks, respectively. Larvae from fish or those previously cultivated will molt at 35 C in a complex culture medium. *P. decipiens* and *Anisakis marina* have produced eggs *in vitro*. *A. marina* eggs from cultured females produced viable larvae. Freshness of the larvae used to initiate cultures is considered a major factor in success.

The histochemistry of the composition and structure of *P. decipiens* cuticle in larvae from fish and cultures has been defined; experiments have demonstrated that the processes of cuticular deposition and ecdysis are independent. It has been postulated and evidence provided that a neurosecretory mechanism controls ecdysis; it has also been shown that this system can be stimulated by an insect hormone and a synthetic analog.

Larvae in vitro, in fish flesh, and in fried fish fingers, do not survive heating to 60 C for 1 min. The recommended time and temperature found in Japanese and European literature for freezing fish to kill anisakine larvae is −20 C for 24 h; however, some North American species survive after 52 h at this temperature.

As experimental animals the anisakine nematodes have distinct advantages and disadvantages. They are plentiful if one has direct access to their natural hosts; the stomach of one Weddell seal may supply as many as 500 adult nematodes, and a single female worm contains an estimated 750,000 ova. A single cod fillet may contain more than 50 infective larvae.

Unfortunately the material does not store or travel well. Numerous poor or negative results in physiological experiments can be attributed to use of shipped parasites. To date, no complete life cycle of an anisakine nematode has been established in convenient experimental hosts. Yet if fresh material is available these parasites have the advantages of relatively large size and abundance, wide temperature tolerance, a variety of hosts in which the worms may develop, and interesting evolutionary divergences among anisakine species and genera; moreover, they can be successfully cultured, in contrast to most other nematode parasites of mammals.

Some experiments now in progress include basic helminthology, carbohydrate and amino acid analyses of tissue and fluids (*10, 32*), behavioral characteristics and adaptations (*27*), and cultivation of the adult and larval stages of anisakines (*23, 29*) as well as work with practical aims such as prevention or diagnosis of human disease.

This paper reviews two aspects of experimental work with anisakines: development in cultures and determination of temperature tolerances. However, the need to establish anisakine life cycles in the laboratory must be stressed.

EXPERIMENTAL HOSTS AND CONTROLLED LIFE CYCLES

Establishment in the laboratory of life cycles of several genera of anisakines from different definitive hosts will remove uncertainties associated with the species, stage, and age of experimental material derived from the field. It may also define the role of the various anisakines as etiologic agents of human disease, add comparative data to the establishment of taxa, and reduce the delays and unpredictable quantities associated with the collection of field material.

Worm penetration studies are one example of the inadequacy of field material for experimental work, especially in laboratories distant from the source. Houwing (*13*) at Ijmuiden, The Netherlands reported that 100% of the fresh *Anisakis marina*[2] he collected would penetrate agar butts in vitro, whereas Ruitenberg, working at an inland laboratory (*28*), reported that, at most, 43% of larvae from the same source would penetrate media. Ruitenberg also selected only rapidly moving larvae; thus only larvae that were the least able to adapt to their environment may have been selected rather than those that were resting through a period of stress. When laboratory-cycled material becomes available, it will at least be possible to test whether the experimental material is still infective.

CULTIVATION

All stages of the anisakine life cycle—egg, larvae, and adults—have been used to initiate cultures. Complex

[1] *The fifth of five papers presented at the symposium "Anisakiasis: A New Disease from Raw Fish," held under the auspices of the New York Society of Tropical Medicine on 14 March 1974 at Rockefeller University in New York City.*

[2] *The name* Anisakis marina *is being maintained here to provide continuity with the European literature.*

culture media are not needed for egg development; water or seawater suffices. In the laboratory it may be necessary to obtain eggs free of microbial contaminants. *Phocanema decepiens* eggs, in common with the eggs of many other ascarids, are sticky and firmly adhere to container walls, which makes them difficult to wash to dilute contaminants to extinction. Moreover, axenizing solutions such as 0.12% glutaraldehyde, 0.4% formalin, 1.33% Zephiran Chloride® [2], or 0.05% sodium hypochlorite halt egg development. Anisakines are more susceptible to chemical disinfectants than some other ascarids. The eggs will develop in the following antibiotics at one and two times the recommended concentration for tissue culture: gentamicin, penicillin, streptomycin, neomycin, amphotericin B, and mycostatin[3].

CULTURE FROM EGGS

Egg development, hatching time, and optimal incubation temperatures vary among species and strains. Kobashi et al. (*19, 20*), reported that unspecified female *Anisakis,* associated in the stomach of the blue-white dolphin with males specified as *A. simplex,* produced eggs which developed in seawater, or physiological saline, but not in 0.5% formol agar. Eggs from individual female worms were incubated separately at temperatures from 2 to 27 C. Table 1 shows the time required for hatching at various temperatures. Two strains are evident: one adapted to high temperatures and one adapted to lower temperatures. Two morphological types of female worms yielded these eggs. One female type was slender-bodied, with the vulva in the anterior portion of the body; the other was stout by comparison, with the vulva in the posterior half of the body. However, both strains of eggs were derived from each morphological type of female worm. This experiment further clouds the already confused taxonomic distinction between *A. simplex* and *A. typica* which Davey (*2*) had seemingly resolved by associating *A. typica,* the species of the stout females, with warmer climates.

TABLE 1. *Temperature tolerance of* Anisakis *eggs*[a]

Temperature (C)	Days to hatching Strain	
	Low temperature	High temperature
2	34	nd[b]
7	14	nd
17	5	11
27	nd	3
37	nd	nd

[a]Compiled from Oshima (*26*).
[b]nd = No development or hatching.

CULTIVATION OF NEWLY HATCHED LARVAE

Some cultivation experiments have been done with anisakine larvae hatched from eggs. McClelland (*22*) and McClelland and Ronald (*23-25*) reported the long-term cultivation, growth, and molting of the hatched larvae of *P. decepiens* (*Terranova decepiens*) and *Contracaecum osculatum* in a mixture of Eagle's minimal essential medium and 20% fetal calf serum. Table 2 indicates the extent of the in vitro larval growth of *P. decepiens.* The average length of larvae at hatching was 140 μm; the average length after 52 weeks at 15 C was 31.1 mm. Growth commenced soon after spontaneous ecdysis of the first stage larval cuticle. The intestinal caecum first appeared in larvae that were 6 weeks old and approximately 2 mm in length. There was no development of the genital primordium, yet overall growth was indicated by an almost constant ratio of the anterior extremity of the genital primordium to the overall length of the nematode. No molts were observed during these 52 weeks. These observations from hatching through 8 weeks of cultivation at 15 C have been confirmed[3].

The freshly hatched larvae of *C. osculatum* were an average of 382 μm in length. They were induced to exsheath with 0.05% sodium hypochlorite (*1*). Their growth pattern is documented in Table 3. The larvae lengthen to 6.5 mm in 32 weeks, the ventricular appendix was present at hatching, the intestinal caecum had developed after 2 weeks of cultivation, and the extent of the overall growth of the nematode was approximately 20-fold in 22 weeks.

The appearance of the ventricular and intestinal appendages so early in the development of these two species supports Janiszewska's (*14*) claims that she could identify larval forms of anisakines from fish as to their genus (but not species). McClelland (*22*) differentiated the position of the excretory pore, a characteristic of taxonomic importance, as early as the time of hatching in both *C. osculatum* and *P. decepiens.*

CULTIVATION OF LARVAE FROM FISH

Numerous workers have taken anisakine larvae from fish and tried to cultivate them to adulthood under conditions that might simulate necessary stimuli from the definitive host. Martin (*21*) reported attempts to grow ascaroid larvae having an intestinal caecum from larvae which had been obtained from the flesh of smelt, *Osmerus eperlenus.* These larvae were probably *P. decepiens* (*Ascaris capsularia*). Two specimens molted at 38 C in a medium of raw fish, pepsin, and HCl. Grainger (*11*) cultured *P. decepiens* and an *Anisakis* sp. under similar conditions. The worms that molted did so 4-7 days after they had fed. The major change reported in both species was the differentiation of the lip mass into three distinct labia. McClelland (*22*) noted additional changes in *P. decepiens:* the doubling of the four lip papillae and development of the genital primordia to produce a rudimentary ovary, uterus, and vagina. No vulva was produced in his cultures.

Promising results were obtained by Townsley's group (*29*), using larval *P. decepiens* from fresh cod (*Gadus*

[2]*Zephiran Chloride is Winthrop Laboratories trade name for a mixture of alkyl dimethylbenzyl ammonium chloride. The mixture of alkyl groups ranges from C8 to C18.*
[3]*Unpublished data.*

TABLE 2. *The in vitro growth of* Phocanema decepiens[a]

Character	Week 0	1	2	4	6	8	12	18	26	34	52
Total length	140[b]	190	412	977	2,380	3,780	6,270	11,300	15,700	21,900	30,100
Esophagus length	37	44	87	213	395	536	705	929	1,170	1,410	1,960
Ventriculus length	15	23	48	103	203	283	390	522	629	778	1,050
Caecum length					43	74	129	202	270	335	702
Maximum diameter	14	19	29	35	70	93	149	244	354	475	632
Genital primordia position[c]	0.58	0.64	0.64	0.65	0.64	0.61	0.58	0.57	F0.52 M0.57	0.50 0.58	0.52 0.57

[a]Adapted from McClelland (*22*).
[b]All measurements in micrometers.
[c]Distance from anterior end as ratio of total length.

TABLE 3. *The in vitro growth of* Contracaecum osculatum[a]

Character	Week 0	1	2	4	6	10	16	22
Total length	382[b]	542	901	1,390	2,020	2,980	4,180	6,000
Esophagus length	77	105	160	231	376	507	579	722
Ventriculus length	13	16	24	34	50	63	81	80
Vent. appendix length	70	81	140	224	351	506	589	693
Caecum length			15	79	168	231	283	387
Maximum diameter	19	26	41	62	91	148	187	243
Genital primordia position[c]	0.61	0.59	0.59	0.59	0.62	0.62	0.57	0.52

[a]Adapted from McClelland (*22*).
[b]All measurements in micrometers.
[c]Distance from anterior end as ratio of total length.

TABLE 4. *Some histochemical reactions of* Phocanema decepiens *cuticle*[a]

Histochemical test	Cuticle from larvae in cod muscle: Cortex[b]	Matrix	Fiber layer	Basal lamella	Cuticle from larvae in vitro at 35 C: Cortex	Matrix	Basal lamella
Periodic acid schiff PAS (Carbohydrate)	±	±	+	±	±	±	±
Hyaluronidase	Fast	Fast	Fast	Fast	Fast	Fast	Fast
Mercury bromphenol blue (Protein)	Blue	Blue/red	Blue	Blue	Blue	Blue/red	Red
Dimethylaminobenzaldehyde nitrite (Tryptophan)	−/+	−	−	−/+	−/+	−	−
Sakaguche (Arginine)	++/+	++	++	+	+	++	+
Van Gieson (Collagen)	+	++	+	+	+	++	+
Collagenase	Fast/Labile	Labile	Labile	Labile	Fast/Labile	Labile	Labile
Dihydroxy-dinaphthyldisulfide (-SH groups)	+++/++	++	+	+++	+++	+++	++
Performic acid-alcian blue (Keratinization) (S-S bonds)	+++/++	+	+	++/+	++/+	+	+
Osmium fixation	++/+++	+	++	+/++	+/++	+	+
Sudan Black B (Lipids)	++	+	++	+	+	+	+
Pyridine ext. and Sudan black B (Lipid confirmation)	++	+	++	+	+	+	+

[a]Adapted from Kan and Davey (*9*).
[b]Where the reactions differ, the symbols to the left of the/indicate outer cortex; those to the right, the inner cortex.

morhua); they reported maturation of both sexes and development of eggs in females. The medium used was human tissue culture medium 199, with beef embryo extract, glucose, and fresh liver extract. In female worms the development of gonadal tissue began two weeks after molting and was accompanied by a rapid size increase, approximately one-third the worms' overall length. The males did not show the size increase, but the tail recurved and became dorsoventrally flattened. Copulation was not observed and the viability of the eggs produced in vitro was not tested.

A brief report by Van Banning (*30*) described the most successful rearing of *A. marina* to date. The cultivation medium consisted of an acid-digested liver extract and citrated beef blood. The larvae molted within 4 days after introduction into culture medium. The final maturation of the nematodes took 1 week and was accompanied by a thickening of the worms; this maturation occurred 26 to 98 days after molting. The problem of insufficient fertilization was overcome by adding cultured mature males to cultures containing just-maturing females. The length of the mature males was between 3.5 and 7 cm

and of the females between 4.5 and 15 cm. Eggs produced in vitro hatched, yielding viable ensheathed larvae.

The major similarity between the successful cultures of Van Banning (*30*) and Townsley's (*29*) group is that both used absolutely fresh fish as a source of anisakine larvae. Those workers reporting molting without maturation worked with admittedly aged material.

In a series of reports on culture of *P. decepiens* from cod fillets, Davey and Kan (*4-9, 16, 17*) described in detail the cuticle, molting, and ecdysis. They also postulated that ecdysis is controlled by a neurosecretory mechanism. The larval cuticle consists of a single layer of longitudinal fibers resting on a basal lamella, a matrix with two faintly osmiophilic bands in a less osmiophilic medium, and an inner and outer cortical layer. The new cuticle formed during molting is produced by the hypodermis and arises through three successive waves of condensation at its outer edge. The central layer splits into three osmiophilic bands in a less osmiophilic matrix. Some of the positive histochemical reactions of larval cuticle and of cuticle formed in vitro are summarized in Table 4. From these reactions, it is evident that collagenase-labile protein is the major constituent of both cuticles. Protection against digestion is gained by formation of disulfide bonds. The cuticle was not digested by trypsin, pepsin, or papain; no evidence of tanning was found.

In vitro experiments showed that secretion of new cuticle is accompanied by typical changes associated with hypodermal and muscle secretory cells: enlargement of the nuclei, nucleoli, mitochondria, and an increase of cytoplasmic and nucleolar RNA. Osmiophilic material from the muscle cells appeared to be secreted into the hypodermis during cuticular deposition. Formation of a new cuticle will occur in the absence of nutrients in a saline solution at 35 C; however, a richer growth medium is necessary for ecdysis.

Ecdysis follows cuticular deposition, after 3.5 to 6 days of culture in a complex medium. Histochemically, ecdysis may be monitored by testing for leucine aminopeptidase (LAP), an enzyme which is found in the excretory gland and is then released into the so-called exsheathing fluid that separates the old and new cuticles. In saline-incubated controls, LAP is released on day 3, but release is not accompanied by synthesis of enzyme, and ecdysis does not occur. On the other hand, nematodes incubated in complex culture medium show an increase of LAP activity in the excretory gland on the first 2 days of cultivation, with release and appearance of LAP activity between the old and new cuticle. A neurosecretory product positive to paraldehydefuchsin is produced by cells of the dorsal and ventral ganglia of the nerve ring (more apparent in the ventral ganglia cells). This synthesis reaches peak activity on day 3 of cultivation. The release of a neurosecretory product coincides with the release of LAP by the excretory gland and is also thought to increase LAP synthesis by the excretory gland. To test this hypothesis nematodes were treated surgically (*9*). With their excretory gland partially exposed, they were placed in a culture medium and treated with extract of the heads (including the nerve ring) of nematodes cultured for various periods. The head extract of worms cultured for 3 days produced a marked increase in LAP production over a 3-h period. Head extracts of worms cultured for 1, 2, 4, or 5 days, or extracts of body wall produced only a weak reaction.

Davey's latest report (*7*) states that saline-incubated nematodes may be induced to ecdyse after 2.5 days if juvenile hormone or a high concentration of farnesyl methyl ether is added to the saline. Nematodes treated with these compounds in normal cultivation medium do not ecdyse, due to the premature release of LAP. Evidence is given that these compounds act by stimulating the neuroendocrine system to produce paraldehyde-fuchsin-positive material.

In summary, Davey and Kan have defined histochemically the structure and composition of *P. decepiens* cuticle in larvae from fish and cultures, demonstrating that the processes of cuticle deposition and ecdysis are independent; they have postulated and provided evidence that a neurosecretory mechanism controlling ecdysis exists; and they have shown that this system can be stimulated by an insect hormone and by a synthetic analog.

TEMPERATURE TOLERANCE

To assure the safety of fish eaten by the consumer the temperature tolerance of anisakine larvae has been studied. Most, but not all, fish are heated before eating in the U.S.; in The Netherlands, herring that is to be eaten raw must first be frozen. Yet, neither country is totally free of anisakiasis.

Heat

Vitzthum (*33*) reported the emergence of ascaroid nematodes from a marinated cod fillet boiled for 20 min. Since his report, no one else has confirmed the ability of

TABLE 5. *Heat tolerance of anisakine larvae*

Temperature (C)	Maximum larval survival time	Genus	Source
60	1 sec	*Anisakis*	Kawada (*15*)
55	10 sec		Houwing (*13*)
50	10 sec	*Anisakis*	Van Thiel et al. (*31*)
45	78 min		Kawada (*15*)
			Khalil (*18*)
40	57 h	*Phocanema*	Ronald (*27*)
45	30 min		
50	10 min		
60	1 min		

TABLE 6. *Heat tolerance of anisakine larvae in fish*

Temperature (C)	Maximum larval survival time	Genus	Source
50	10 sec	*Anisakis*	Van Thiel et al. (*31*)
55	10 sec		Houwing (*13*)
40	57 h	*Phocanema*	Khalil (*18*)
45	30 min		Ronald (*27*)
50	10 min		
60	1 min		

anisakine larvae to withstand temperatures over 60 C. Tables 5 and 6 summarize the confirmed data on the temperature tolerance of *Anisakis*-type and *Phocanema*-type larvae from fish. This evidence was obtained by placing the isolated nematodes in incubation solutions and then applying heat; by adding the nematodes to already heated solutions; or by heating the nematodes in fish. Ronald (*27*) also reported similar data on larvae in moist chambers and evaluated the insulative properties of fish flesh.

The data in Tables 5 and 6, derived by different methods, are closely comparable and place the upper limit of 60 C for 1 min as the minimum necessary to kill all anisakine larvae.

TABLE 7. *Survival of anisakine larvae in fired fish fingers*[a]

Temperature (C)		Time (min)	Number of Larvae		
Initial	Final		Dead	Sluggish	Active
–13	55	4.00	10	0	0
–10	55	5.00	3	3	4
–7	55	4.25	7	3	—
4	>60	5.45	10	0	—

[a]Adapted from Davey (*3*)

Table 7 summarizes the data of Davey (*3*) on the temperature tolerance of *A. marina* larvae in fried fish "fingers." Larvae and a thermistor were implanted in a cavity of a fish finger, covered with fish flesh, sealed with batter, and refrigerated or frozen overnight. The fish fingers were then fried in shallow fat until the thermistor reached a predetermined temperature. The nematodes were transferred immediately to warm saline, observed for 1 h, and classified as dead, sluggish, or active. These data from fried fish fingers further support the other temperature studies.

Examination of the tabular data indicates that fish should be cooked so that the internal temperature reaches or exceeds 60 C for at least 1 min; this will assure the consumer that all anisakine larvae are killed.

Cold

Anisakine larvae are capable of surviving extended periods of refrigeration that easily exceed the useful shelf-life of fresh fish. Ronald (*27*) reported that larvae kept refrigerated in a physiological solution at 0 C survived for 133 days.

TABLE 8. *Cold tolerance of anisakine larvae in fish*

Temperature (C)	Maximum larval survival time (h)	Genus	Source
–5	144	*Anisakis*	Gustafson (*12*)
–10	288		
–17	10		
–5	96	*Phocanema*	Ronald (*27*)
–10	17		
–20	16.5		
–20	52	*Contracaecum*[a]	

[a]Unpublished data.

Table 8 shows the maximum survival time of anisakine larvae exposed to freezing temperatures in situ in fish flesh or on the mesenteries. Survival at –20 C duplicates the temperatures of home freezers or modern refrigerator freezing compartments, and is similar to the temperature of the common commercial block freezing apparatus. Ronald (*27*) used the criterion of fluorescence under U.V. light to determine viability; all other workers used the criterion of motility after thawing and recovering the larvae.

Ruitenberg's (*28*) translation of The Netherland's Green Herring Law is: "All herring to be sold as lightly salted herring must be frozen in such a way that the herring has reached a temperature of –20 C within 12 h. Furthermore, the herring so frozen should be stored during a period of 24 h in such a way that this herring is kept at a temperature of *not less* than –20 C during this period." The data upon which this statement is based indicate a mistake in translation. The last part should read . . . "the herring is kept at a temperature of *not more* than –20 C during this period." Our studies indicate that this regulation may not be sufficient to inactivate the variety of living anisakines found in the fish products of North America.

SMOKING AND MARINATION

The effectiveness of other methods of preserving fish such as smoking and pickling have been studied. Oshima (*26*) reviewed the marinating and pickling of fish in Japan, and Ruitenberg (*28*) reviewed marinating and smoking procedures in The Netherlands. Comparison of data is difficult because of the different methods used in each country. In general, smoking and marination procedures are not sufficient to kill anisakine larvae. The possible exception is dry salting, which will kill anisakines if the salt reaches all parts of the flesh in concentrated form.

CONCLUSION

Although only some of the work on experimental anisakiasis has been reviewed, the examination of cultivation and temperature tolerance of anisakine nematodes supports the author's conviction that the establishment of laboratory life cycles is necessary to provide experimental material in the quantities necessary for reproducible results.

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Practical Implications of Injured Microorganisms in Food

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ABSTRACT

Recognition of sublethally impaired microorganisms is essential to practical interpretations of data in food microbiology. Sublethal injury induced by exposure to environmental stress often is demonstrated as a loss by the microorganism of one or more abilities to function characteristically under conditions that are satisfactory for untreated cells. Various treatments related to food processing such as heat; cold; freezing-thawing; freeze-drying; moisture reduction; irradiation; and exposure to food environments, sanitizers, or preservatives may induce sublethal damage in cells or spores. Many cellular modifications may be observed in injured cells. Included are susceptibility to selective agents and antimicrobials, leakage of intracellular material, and modified metabolic activities. Injury is characterized by the capability of the microorganism to return to normalcy during a resuscitation process in which the damaged essential components are repaired. Sublethal damage in spores must be considered specially because of the complexity of the spore entity and its characteristic high resistance to stress. Extent of sublethal damage and mechanisms of injury and repair are varied and related to conditions of stress and resuscitation. Knowledge of sublethal injury is indispensable in evaluating laboratory data, in developing or modifying food processes, and in preserving culture activity.

When can we be confident that no one will isolate salmonellae from a food product that has been declared free of detectable salmonellae in a specific series of examinations? Why do some dairy starter cultures require special treatment to be active after cold storage? When are numbers of viable staphylococci truly no greater than the measured small population observed in a food ingredient? Why do apparently sterile items upon protracted storage show evidence of viable bacilli or clostridia?

Why do these apparently absent, inactive, or dead microorganisms suddenly appear? Spontaneous generation? Would any follower of Pasteur accept that? Reincarnation? Only a fanatic pseudomonad would claim to have returned as a vibrio!

No more complex explanation is required than to state that all of these observations may indicate the presence of injured cells. Simply, any of these observations may represent sublethal damage that results in injured microorganisms which can undergo subsequent resuscitation where damaged components are repaired and the cell recovers its normal capabilities.

A great interest in damaged microorganisms has been evident in recent years. Much of the research has originated in food microbiology laboratories and has been directed toward practical implications of the findings. However, the research has often been of fundamental importance to the entire field of microbiology and thus results have been published in a variety of journals, many of which are not always read by those interested in applied science. Hence, this paper is an attempt to summarize some of the practical implications of cell injury as they relate to food microbiology and food technology. The paper is not comprehensive in consideration of the literature but rather will be selective in examples of the associated phenomena.

Sublethally impaired microorganisms are important in each of the major areas of food microbiology, i.e., in food preservation and spoilage, in food safety and consumer protection, and in food manufacture and culture propagation. Some essential considerations could include use of damage to enhance lethal action of a processing treatment, minimizing damage to preserve culture activity in food fermentations, predicting effects of product formula modification on subsequent microbial damage and survival, and eliminating inadequacies within existing and proposed methodology to detect and enumerate specific microorganisms.

Historically, many investigators identified certain media and cultural conditions as superior for growth of certain microorganisms retrieved from various environments. Examples of this would be the observations of Nelson (*63, 64*) and Heinmetz et al. (*38*) that coliform bacteria after exposure to thermal and chemical stresses are less productive on selective media than are unstressed cells. Similar observations were made with freezing treatments (*37*). Development of apparent cultural inadequacies observed after exposure of bacteria to environmental stresses eventually was categorized as cellular injury. Straka and Stokes (*94*) identified development of a requirement for complex media components by pseudomonads that had been exposed to a freezing stress. Busta and Jezeski (*15*) observed a loss in salt tolerance of staphylococci after a sublethal heat shock. Moss and Speck (*58*) detected a nutritional need for peptides by lactic streptococci that were stored at low temperatures.

All of these observations had a similar theme. After exposure to some sublethal treatment that applied an environmental stress to the cells, bacteria lost a characteristic ability to grow normally under conditions that were satisfactory for untreated cells.

Injury may be manifested as the inability of bacteria to

form colonies on a defined minimal medium while retaining the colony forming capability when complex nutrients are supplied. It may be observed as the inability of the organism to multiply in a liquid or solid medium that contains a selective agent that has no inhibitory action on unstressed cells. Evidence of damaged cells may be in the form of an extended lag phase producing slow growth with accompanying slow accumulation of end products. Regardless of the method of evaluation, injury is observed when cells that survive a given stress lose some of their characteristic growth capabilities.

TREATMENTS RELATED TO FOOD PROCESSING THAT INDUCE CELL INJURY

Numerous treatments and food processes are employed to inactivate or control growth and survival of microorganisms. Most agents that have the potential to destroy specific microorganisms also can, when applied at sublethal levels, damage the cells.

Heat

Processing procedures that involve use of elevated temperatures may stress microbial cells or spores. Relatively low temperatures, as found in the pasteurization (milk) or subpasteurization range can injure *Staphylococcus aureus* (*16*). Similar temperatures associated with concentration and dehydration processes also could generate such stresses. Elevated temperatures of blanching or cleaning have the same potential.

Heat-induced injury of the following species has been reported: *Staphylococcus aureus* (*16, 93*), *Streptococcus faecalis* (*11, 19*), *Salmonella typhimurium* (*18, 30*), *Escherichia coli* (*79*), *Aerobacter aerogenes* (*95*), *Pseudomonas fluorescens* (*35*), *Vibrio marinus* (*36*), *Bacillus subtilis* (*51*), *Clostridium botulinum* (*67*), and *Candida utilis* (*100*).

Thermal processing at conventional and ultrahigh temperatures damages bacterial spores from *Bacillus subtilis* (*14, 23, 89*), *Clostridium perfringens* (*9, 17, 22, 77*), *Clostridium sporogenes* (*28, 29*), and *Clostridium botulinum* (*4*).

Cold

Reduced temperatures above O C are used frequently to preserve foods. These refrigeration-level temperatures can induce injury in *S. aureus* (*45*), *Streptococcus lactis* (*90*), and *C. perfringens* (*99*).

Freezing and thawing

Processes at temperatures below O C include freezing preservation of food, concentration of liquids, and production of frozen desserts. Stresses of freezing and thawing can induce injury in numerous bacteria such as *E. coli* (*53, 59*), *Salmonella anatum* (*47, 69*), *S. lactis* (*58*), *Shigella sonnei* (*62*), *S. faecalis* (*55*), and *Pseudomonas fluorescens* (*7*). The extensive information on freeze-injury of bacteria has been well reviewed by Ray and Speck (*75, 76*) and by Fennema, Powrie, and Marth (*24*). Unfortunately, to date, the specific influences of the independent stresses of thawing and freezing have not been well differentiated.

Freeze-drying

Removal of moisture from food or biological materials in the frozen state is a common but sophisticated and gentle preservation technique. Nevertheless the many microorganisms that exhibit damage from the resultant stress include *S. anatum* (*70*), *E. coli* (*85*), *Streptococcus thermophilus* (*54*), and *S. aureus* (*8, 27*). Thus food specialties prepared by freeze-drying could contain injured microorganisms, and cultures for food manufacture or for laboratory use preserved by this procedure may contain damaged cells. Unfortunately, to date the specific influences of the individual stresses of freezing, dehydration, and rehydration have not been well differentiated.

Moisture reduction

Removal of part or most of the available water occurs in many food processes that utilize elevated temperatures, low pressures, and dehydration equipment such as agitators, evaporators, and spray and roller driers. Food products such as dried milk contain bacteria that appear to be injured (*72, 73*).

Incidental dehydration during aspiration or during chance drying on equipment also may generate a damaging effect (*92, 101*). Reduction of water in intermediate moisture foods through addition of humectants damages bacterial cells (*42*). This damage may be caused by mechanisms similar to those observed in other types of dehydration or freezing injury.

As with freezing and thawing and with freeze-drying and rehydration, stresses involved with removal of water have not been well differentiated from those associated with the rehydration step which could be the more influential of the stresses involved.

Nutritional environment

Placement of cells into a new environment may generate stresses that result in injury. A growth environment of new or different substrate concentrations or osmotic strengths could produce stresses that resemble dehydration or rehydration (*42, 73*). Initiation of growth in a fresh complex medium may produce cells that respond to selective or minimal media in a fashion that resembles injury (*82*). Placement in a spent medium may expose cells to end products such as acids that promote damage (*52*). Incidental exposure to air may influence the apparent amount of injury (*31*). Exposure to diluents of various types and especially distilled water may injure cells (*40, 50*). It is reasonable to suspect that cells exposed to starvation conditions that are apparently lethal might also display sublethal damage if survivors were determined appropriately.

Irradiation

Use of UV irradiation for sanitization, incidental irradiation of surfaces in sunlight, or use of gamma irradiation for food preservation have potential for

generating repairable sublethal damage in microorganisms associated with foods. This may be best demonstrated in radiation-resistant strains (*21*).

Sanitizers

Chemicals employed to sanitize items in the food industry readily induce injury in bacteria if the sanitizers are used under conditions and at concentrations that promote sublethal damage and minimize complete inactivation of the cells (*81*). Injured cells could readily find routes into food systems and conditions so they could produce misleading test results and other potential problems after repair.

Preservatives and acidulants

Food additives and end products of food fermentations that are preservatives or acidulants frequently serve as inhibitors of unwanted growth rather than as lethal agents for unwanted microorganisms. Exposure to acids in combination with other stresses damaged *S. aureus* (*52*), whereas exposure to alkali altered spores of *C. perfringens* (*22*). There is no reason to believe that exposure of some microorganisms to various preservatives could not induce cellular damage.

Combinations of treatments and interactions of stresses

Some processing treatments such as freeze-drying are by definition combinations of several stresses. Unfortunately, investigations on cell injury often neglect the measurement of interactions and synergistic effects among the various individual components of the treatment. The presence of low levels of NaCl in the heating medium reduces the amount of heat injury of *E. coli* measured as increased susceptibility to NaCl in the plating medium (*84*), whereas sorbic acid enhanced thermal injury in *C. utilis* (*100*). Freezing in combination with low pH enhanced the extent of injury in *S. aureus* (*52*). Combinations of heat treatments and several humectants in the heating or growth media resulted in various responses that indicated cell injury and the responses were dependent on the type of humectant and the species of bacteria (*41*). Once can only anticipate considerably more interactions to be documented in future comprehensive studies.

CELLULAR CHANGES INDUCED BY STRESS

After sublethal damage, injured cells often are observed or identified by their inability to proliferate under specific previously productive conditions. This inability may be measured by lack of colony formation in solid media, absence of turbidity in broth media, or low production of end products from appropriate substrates. A host of cellular changes have been implicated and related to depressed growth capabilities of injured cells.

Often injury is observed as an increased or new sensitivity to selective agents, antimicrobials, or similar substances in the growth medium for the damaged cells. Salt tolerance is lost by *S. aureus* (*16*), *B. subtilis* (*51*), *E. coli* (*84*), and *S. typhimurium* (*18*). Deoxycholate inhibits injured *E. coli* (*82*). Actinomycin D can penetrate and act on damaged *S. anatum* (*71*). Heat-altered spores of *C. perfringens* and *C. botulinum* are susceptible to lysozyme-mediated germination (1, 4). Frozen *S. facealis* become sensitive to sodium azide (*55*). One possible interpretation for these newly developed sensitivities would be cell membrane or cell envelope modification in the damaged cells.

Injured cells frequently lose some of their cellular material through leakage into the surrounding medium. Frozen cells of *E. coli* release amino acids, small molecular weight ribonucleic acids, and peptides (*75*). Heat-injured *S. aureus* cells release potassium, amino acids, proteins, and 260-nm absorbing material (*6, 44*). Similar patterns have been observed in other damaged bacteria. Release of lipopolysaccharide indicated damage to the outer membrane, was correlated with sublethal injury, and preceded death in heated *E. coli* (*39*). Obviously when sufficient essential cellular material is lost and not restored by the cell operating under appropriate conditions, damaged cells are not able to proliferate in the normal fashion.

Macromolecules within the cell are modified by stresses. Ribosomal ribonucleic acid was degraded in heated cells of *S. aureus* (*6, 44*) and in *S. typhimurium* (*96*). Although several investigators have reported on thermally induced DNA breaks (*13*) and DNA repair (*102*), they have related these changes to death rather than injury. However, recently DNA breaks were correlated with heat injury of salmonellae that responded negatively to complex nutritional media (*30, 32*). Disruption of these essential components in damaged cells would be totally disabling if not restored to permit growth.

Metabloic activities associated with the cell are affected by damaging stresses. Sublethally heat-injured *S. aureus* have decreased catabolic capabilities and reduced activities of selected enzymes of glucose metabolism (*12*). Glucose transport in heat-damaged *S. typhimurium* was altered (*66*). A membrane proteinase of *S. lactis* was inactivated by low temperature storage (*90*). Many other modified metabolic activities and systems may be correlated to injury in future work.

Most cellular modifications identified and correlated to cellular injury have been measured coincidentally with the observed injury. Added research is needed to clearly establish the cause and effect relationship. Regardless of the lack of absolute identification of the specific modification(s) that do regulate expression of injury, it is evident that the injured cell has undergone numerous changes that might be interrelated and the cell requires special treatment to return it to its normal state. The return to normalcy has been termed repair or resuscitation and has been used extensively to study and identify specific cellular components that have been adversely affected by the stress.

RESTORATION OF LOST CAPABILITIES

Cells are classified as injured rather than dead when they are damaged but have the capability to function in an unrestrictive environment and restore a normal physiological state concommitant with initation of growth and cell division. By definition, the evidence for damage must disappear upon cell division to support the observation of injury and rule out the possibility of permanent changes in the form of mutations. Restoration of the undamaged state has been identified as a recovery or repair process but probably is best characterized by the term resuscitation (*5*) because the cells are revived (or made operative) from apparent death.

The resuscitation process clearly must be related to some or most of the cellular changes that occur after a sublethal treatment that impairs certain activities of the microorganism. Many of the cellular modifications are reversed or losses are restored to the normal state during an incubation period that precedes any evidence of cell division. Ribosomes that have been degraded during a heat treatment are regenerated (*44, 88, 96*). Phospholipids are synthesized during the recovery period (*98*). Protein synthesis appears to be necessary in repair of some frozen, freeze-dried, or heated cells (*75, 76, 85, 97*). The repair process is dependent upon energy synthesis apparently in the form of ATP (*69, 71, 74*). Heat-induced single strand breaks must be repaired to prevent death (*30, 102*). Synthetic activities in one strain damaged by a specific stress may be unique and not required in other organisms damaged by different stresses.

Recognition of the presence of potentially injured microorganisms and the subsequent value of a resuscitation period in the isolation and enumeration procedures is evident from the literature. Most salmonellae isolation procedures involve pre-enrichment steps which allow resuscitation. A rapid method to test for *Enterobacteriaceae* in dried foods utilizes a restoration treatment (*60*). A recently published plating procedure for injured coliforms utilizes a resuscitation period before exposue to the selective agents (*91*). In some instances, certain selective media are not antagonistic to damaged cells and permit repair and subsequent colony formation by cells that are essentially all damaged (*34*). Unfortunately many laboratory procedures continue to disregard the presence of sublethally impaired microorganisms in analytical samples.

SPECIAL CONSIDERATIONS OF SPORES

The spore is dramatically different from the vegetative form of the microorganism. Spores are formed under special conditions that prompt this cellular differentiation by vegetative cells. Spores have special characteristic components that are not present in cells. Generally resistance of spores to stresses such as heat is considerably greater than that of vegetative forms. To demonstrate viability, the spore must initiate germination, germinate, lose its spore-like characteristics, grow out into a vegetative cell, and then proceed to proliferate in this form. These many different operations performed during spore formation, germination, and outgrowth when coupled with high resistance to stresses make it necessary to consider spore injury separately.

The influence of injury on apparent survival and viability of spores has been recognized since 1923 (*83*). Any measurement of resistance depends on actual or true survival and "the ability of the surviving spore to germinate, reproduce, and lead to a sufficient number of generations to be recognized as a survivor under the subculture conditions used" (*83*). Several early workers demonstrated that subculture media dramatically influenced the apparent survival (*20, 56, 57, 63*). These were obvious indications of damaged spores.

Heat-induced susceptibility to antagonistic materials in the culture media was evident in heated spores of clostridia (*26, 65*) and bacilli (*61*). The antagonistic effects of salts (*77*) and of antibiotics (*25*) on heated spores further document the occurrence of injury. Chemical treatments also generate apparent damage (*78*).

When *B. subtilis* spores were exposed to ultrahigh temperatures for short times, heat injury was observed by detecting damaged survivors through addition to the medium of a non-nutritive germinating agent, calcium dipicolinate (*23*). Injury resulted because a specific germination system was inactivated in the spores but the spores remained viable if germinated in other ways (*2, 3, 14*).

Apparent loss of viability after severe heat treatments of clostridial spores was reversed with the addition of lysozyme to a conventional medium (*17*). This has been confirmed in heat-injured *C. perfringens* spores (*1, 22*). Lysozyme apparently replaced the normal lytic activity of germinating spores which had been altered by the treatment.

Outgrowth also may be influenced by a heat treatment. The frequently observed heat-induced shift in optimal temperature for maximal enumeration of surviving spores was related to outgrowth capabilities rather than germination (*68*).

Recently Busta, Baillie, and Murrell (unpublished data) observed a heat-induced requirement for sucrose in the recovery medium of adolescent or immature spores of a *Bacillus sp.* This requirement for sucrose disappeared after the spores had germinated and reached the first division and therefore the damage must involve some process in outgrowth or beyond.

With data accumulating at an increasing rate, heat damage in bacterial spores is being considered in the study of mechanisms of heat resistance (*33*). Presumably, currently active investigators evaluating thermal processes are also aware of the significance of heat-damaged surviving spores. Obviously, any research on inactivation of spores by any antagonistic agents must consider potential injury when there is an apparent lack

of viability. Recovery of *B. subtilis* spores surviving exposure to phenol was enhanced by L-alanine or D-glucose; however, comprehensive evaluations of chemical damage are not evident in the literature.

FACTORS THAT INFLUENCE INJURY

Growth conditions that influence the physiological state and composition of the cell or spore may affect susceptibility of the microbe to damage by subsequent exposure to one or more stresses. Changes in rates of heat resistnace in *S. aureus* could be interpreted as major variations in the amount of heat injury in the survivors; however, the rate of heat injury changed less than four-fold when sampled throughout the entire growth period (*43*). Minimal recovery of heated *S. typhimurium* emphasizes that growth media and conditions influence susceptibility to injury, the way injury is expressed, and the mode of repair by the damaged cells (*32*). Specific nutrients, pH, redox potential, osmolality, water activity, ionic strength, surface tension, temperature, agitation, gaseous atmosphere, culture age, and any other variables of a specific growth system could interact or act upon the structure and functions of the cell, and therefore must be considered for potential influence on the expression of injury.

Most factors that influence cell quality also may have an effect during application of stress. Storage temperature and humidity influenced the amount of injury in freeze-dried *S. typhimurium* (*86*). The extent of freeze-thaw damage in *S. anatum* was responsive to milk components in the suspending menstruum (*46*) as was the apparent freeze-thaw injury of *E. coli* in various foods (*76*). Solutes including phosphate buffers, Mg^{2+}, and sucrose altered the percentages of thermally injured *S. typhimurium* (*49*). Low pH increased damage in freeze-thaw injured *S. aureus* but had little effect on heat injury of the same bacteria (*52*). The pH and NaCl concentration in the heating system influenced the amount of heat injury in *S. aureus* (*10*). Many of the frequently used cryoprotective agents are directed at minimizing the extent of freeze-thaw or freeze-drying damage of cells (*24, 75*).

Furthermore, many of the factors that affect cell quality and growth may influence resuscitation of injured cells. The repair of heat-injured *S. aureus* (*44*), repair of injury in freeze-dried *S. anatum* (*71*), repair of freeze-thaw damaged *S. anatum* (*48*), revival of heated *E. coli* (*80*), repair of dehydration damage in *E. coli* (*101*), minimal recovery of heated *S. typhimurium* (*32*), and interaction of pH and NaCl on recovery of heat-stressed *S. aureus* (*87*) are a few examples of the extensive research and information available on factors that influence the return of injured cells to normalcy.

Antagonistic agents in the form of metabolic end products or food additives or both should have potentially greater activity against some damaged cells. Whether as gases, acids, alcohols, or other end products or incorporated as adjuncts during food manufacture, one could speculate that these materials would have an influence on damaged cells that was greater than on non-stressed cells. As indicated in earlier sections, many antagonistic or selective agents may produce damaging stresses (acid, alkali, sanitizer, NaCl, etc.) or may serve as the specific agents that inhibit damaged cells and are used to detect or define injury (NaCl, deoxycholate, acid, etc.). These latter agents obviously negatively affect repair of injury. With cell injury as with evaluation of inactivation, one must consider all factors that are influential before exposure to the agent, during exposure to the stress, and after exposure to the treatment.

CONCLUSION

With the continuing accumulation of data on damaging effects of sublethal treatments of microorganisms associated with foods, it should be possible to alleviate or at least minimize most of the problems that arise from the presence of damaged microbial cells. Procedures that lead to improper detection, enumeration, identification of given microorganisms should be adjusted so that accurate assessments of actual situations are made. Contamination of foods or food environments with damaged cells that are capable of repairing the injury should be anticipated. Delayed undesirable actions by these repaired cells then could be eliminated or reduced. Reduced growth or activity of contaminants that serve as competitors for pathogens should be indentified and subsequent control of hazardous cells measured. All potential imbalances in ecological relationships, including the associative growth of multiple strain starter cultures, should be re-evaluated after exposure to stress.

Sublethal injury could readily serve as a selection process that would eliminate sensitive forms and promote the domination by resistant forms of various microbial groups exposed to the damaging stress. To date there is no strong evidence to support or promote use of treatments at the sublethal stress level for control of unwanted microorganisms, because unwanted types do not appear to have any special sensitivities to stresses when compared to innocuous types. However, the possible induction of increased sensitivities to selected preservatives, lytic enzymes, or other antimicrobial material should be recognized as a potential major safety factor. Identification and characterization of various fundamental aspects of sublethally impaired cells should give an insight into the mechanisms of lethal inactivation treatments and ultimate death in microbial cells and other biological systems.

Microorganisms used to manufacture and/or preserve specific foods should be handled in an appropriate manner to reduce the deleterious effects of sublethal environmental stresses. An extended lag phase, resulting in delayed growth and reflecting an apparent inability to multiply, might be minimized by medium adjuncts or other procedures that compensate for modifications in nutritional requirements of damaged cells. The potential decrease in genetic stability should also be recognized.

The possible depression of cultural or metabolic activities subsequent to culture preservation or storage regime should be considered whenever starter cultures are used. The influence of stress also must be considered in balancing multiple strain starters. Obviously, an understanding of sublethal damage and resuscitation can do no less than increase the competence of individuals making decisions in food microbiology.

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Report of the Committee on Food Equipment Sanitary Standards - 1975

The IAMFES Committee on Food Equipment Sanitary Standards, known hereafter as the Committee, is charged with the responsibility of cooperating with other interested health organizations and related industries in the formulation of sanitary standards and educational materials for fabrication, installation, and operation of food equipment and to present to the membership those standards and educational materials which the committee recommends be endorsed by the Association.

The purpose of this cooperative program is to aid industry in improving the design, construction, and installation of equipment so that it will lead to easy cleaning and proper functioning when placed into service in food establishments. It is the Committee's further purpose to cooperate with industry in the preparation of standards or guidelines which public health agencies will accept, thereby securing uniformity in the manufacture and nationwide acceptance of such equipment.

The following report will outline the Committee's activities during the past year in working with two health and industry organizations (National Sanitation Foundation's Joint Committee on Food Equipment Standards and the National Automatic Merchandising Association's Automatic Merchandising Health Industry Council) and progress in meeting its purposes and objectives. It is expected these organizations will be the two groups that the Committee will work with during the coming year.

NATIONAL SANITATION FOUNDATION(NSF)

The Committee was represented at the 1975 meeting of the National Sanitation Foundation's Joint Committee on Food Equipment Standards, where action was taken on several proposals; and before the meeting, the Committee reviewed and submitted comments on each draft of these proposals. Since the meeting, the Committee has also reviewed and submitted comments on proposed changes to standards.

Basic criteria C-2—Special devices and equipment

Following a brief review of the durability of materials and finishes requirements of NSF and some problems presented at last year's Joint Committee meeting by a major restaurant chain on durability of NSF approved equipment, a tabulation of questionnaires sent out during the past year to regulatory officials, manufacturers, and user/consultants was discussed. According to the NSF staff most of the responding sectors represented felt the current NSF requirements were adequate. However, some responses noted particular instances where the requirements could be up-graded. The Public Health representatives requested NSF to investigate further and begin work on resolving the particular material problems noted by the questionnaire respondents. NSF acknowledged this request indicating it would also revise its material test procedures.

The NSF Staff reported receiving complaints from regulatory officials of corrosion problems when 430 series stainless steel was used in the fabrication of sinks and ice bins. The Public Health representatives recommended that the use of 430 series stainless steel be eliminated on those pieces of equipment subject to continued wetting and oxidation. They further requested that the Joint Committee and appropriate task committees make these revisions as NSF food equipment standards are periodically reviewed.

The Joint Committee received a report from a county health department asking that data plates be provided on reach-in refrigerators designed specifically for prepackaged bottled products. It was noted that this data plate is necessary as many food service establishment operators have attempted to use these units for the storage of unpackaged products. These units do not meet the NSF Standard No. 7's construction requirements but do, however, meet the Standard's performance requirements. It was the consensus of the Joint Committee that NSF should require data plates on reach-ins designed specifically for prepackaged or bottled products and further that the label should properly identify this equipment and be in a visible location.

The NSF reported that annually a physical evaluation is conducted on listed food service equipment; however, the performance tests are not repeated unless there has been a major design change in the equipment. It was the consensus of the Joint Committee that NSF should immediately institute a periodic retesting program on those items of equipment which require performance testing.

Standards No. 1, 12, 18—Ice storage equipment

The Joint Committee reviewed Michigan's Guidelines relating to the Protection of Potable Iced in Food Service Establishments. Following a review and discussion of the guidelines, the Joint Committee recommended that NSF Standards 1, 12, and 18 be revised to prohibit installation of carbonators and drop-in cold plates in ice bins. However, built in cold plates would be acceptable providing they were an integral part of the wall or floor of ice storage equipment. It was the consensus of the Committee that NSF should establish a task committee to consider these revisions and further to review whether or not covers should be removable or readily removable and the problems associated with hinged and sliding covers.

Standards No. 2 and 4—Food charts and carriers

The NSF Staff noted that presently NSF did not have performance test requirements for insulated food carts and carriers and felt they should be developed. The Joint Committee recommended that NSF start requiring performance testing of insulated food carts and carriers designed to hold hot and cold foods, and that a standards task committee be formed to set the performance, time limits, loads, and ambient test conditions. The Committee noted that the test procedures should be developed for Standard 4 holding carts as well as Standard 2 mechanical and nonmechanical carts and carriers.

The NSF Staff reported that the Foundation continues to receive questions from regulatory officials concerning the construction of certain NSF listed carts, particularly the joints and seams. After considerable discussion regarding the differences in manufacturers' designs, it was concluded that NSF should form a task committee to review the existing requirements and current manufacturers' designs, and report back to the Joint Committee.

Standard No. 3—Commercial-spray-type dishwashing machines

The Joint Committee then discussed the proposed revisions to Standard 3 for Spray-Type Dishwashing Machines. The NSF Staff reported that the standards task committee had not concurred with the Joint Committee's recommendation to require final rinse arms to be *readily removable*. After considerable discussion, the Committee recommended the standards task committee consider revising the requirement to make final rinse arms *removable* and self-aligning. Following a brief discussion of the sound levels, it was recommended that the standards task committee be directed to consider establishing maximum sound levels for commercial dishwashing machines. It is anticipated that the task committee will meet in early Fall and that the revised proposed revisions to Standard 3 will then be forwarded to the IAMFES Committee for review and ballot.

According to the NSF Staff, the comments concerning the proposed revision to Standard No. 3 which this committee submitted following its meeting last year in St. Petersburg, Florida, will also be considered by the task committee.

Standard No. 4—Cooking and hot food storage equipment

According to the NSF Staff, a vertical rotisserie called Souv-Laki Gyros could be fabricated to meet the design and construction requirements of NSF Standard No. 4; but through improper operation, the units could present a significant public health problem as the center portion of the meat roll could be held at incubation temperature for prolonged periods. Consequently, the Public Health representatives recommended there be included in each food equipment standard being revised a section requiring appropriate data from the manufacturer in those instances where a potential public health problem could exist.

Standard No. 8—Powered food preparation equipment

In accordance with a request of the Public Health representatives at last year's meeting of the Joint Committee, recent articles on the role of the meat slicer in the dissemination of salmonellosis were investigated. It was found that improper cleaning and sanitization procedures had been followed and that the slicers involved did not meet NSF design and construction requirements.

The Joint Committee also reviewed the position of the U.S. Department of Agriculture regarding aluminum noting that aluminum is not permitted on wearing surfces, as the black oxides color meat coming in contact with it. The Public Health representatives recommended that NSF discuss further with USDA their position regarding aluminum and that NSF look at the public health aspects of aluminum in food service equipment. Further, the representatives requested that when Standard 8 is reviewed again, revisions be considered regarding the use of aluminum on wearing surfaces of food preparation equipment.

Standard No. 18 and 25—Food and beverage dispensing vending equipment

The NSF staff reported on the comments received from public health representatives regarding the standards presently available for refrigerated drinking water fountains. The responses received were quite varied. Consequently, the representatives requested NSF to contact the industry asking if they were interested in supporting the development of a standard. Following this review, NSF was requested to report its findings to the Joint Committee.

It was reported that the proposed revisions to NSF Standard No. 25 to provide for air gaps only with no copper downstream was still pending. Apparently, the members of industry felt NSF Standard 25 should be consistent with Standard 18's backflow prevention requirements. Further, it was noted that NAMA-AMHIC was reviewing their backflow prevention requirements and that NSF would await the outcome of that review.

Standard No. 33—Cooking equipment exhaust systems

The NSF Staff reported that Standard No. 33 has never been applied due to the difficulty in evaluating some of these exhaust systems and that the standard conflicts with other nationally recognized standards and building codes relating to exhaust systems and that the quantity of air to be exhausted differs from many regulatory agency requirements.

Following a discussion by the Joint Committee members and industry participants present, the Committee recommended that a task force be formed to review and, if possible, resolve these problems. The Committee requested that if it were not possible to resolve these problems, and the task committee recommended Standard 33 be dropped, a report should be directed to the Foundation justifying the task committee's recommendation.

Standard No. 36—Dinnerware

The Joint Committee reviewed the work that NSF had recently completed on "pewter-like" dinnerware. It was noted that this aluminum alloy material could not withstand the abrasion (knife test) specified in Standard 36. Following considerable discussion regarding the advantages and disadvantages of partial NSF listings and the concept of testing based on intended end use, the Joint Committee recommended that NSF should test "pewter-like" dinnerware based on the end use applications. NSF, therefore, will not be required to apply the cutting test to those items of "pewter-like" dinnerware (goblets, cups, saucers and the like) that were not designed to be cut upon.

Plans for future standards

The NSF Staff reviewed the progress that had been made in the development of the standard for Plastic Materials and Components Used in Food Equipment noting that recent contact had been made with FDA and that the standard would be sent to the committee later in the year for their review and ballot.

The NSF Staff reported a proposal to develop a standard for intermittent pyrethrin dispensers and aerosol sanitizer/deodorant dispensers. Following an extended discussion of the public health aspects, the Joint Committee with one opposing vote (IAMFES) recommended NSF proceed with the development of the standard as noted below: (*a*) distribute data to the Joint Committee for their review and information; (*b*) await EPA publishing its position on these dispensers in the Federal Register; and, (*c*) form standards task committee to begin developing standard. The IAMFES committee representative opposed this action because of the manufacturer's intention for such equipment to kill insects on a 24-h basis without adequate safeguards to prevent dying flies from dropping in food during preparation periods.

The Joint Committee reviewed the proposed standard for Supplemental Flooring. The Committee recommended some changes in the proposed standard. NSF will refer these changes back to the standards task committee for their consideration.

According to the NSF staff, some progress had been made toward development of a standard for high pressure cleaning equipment.

Plans for educational program

The NSF staff reviewed the past year's activities of NSF Educational Services and asked the committee for comments on NSF's prospectus for a National Conference of Sanitation Aspects of Food Facility Planning and Plan Review to be held the latter part of 1975.

NATIONAL AUTOMATIC MERCHANDISING ASSOCIATION

The National Automatic Merchandising Association's Automatic Merchandising Health-Industry Council (AMHIC) held its twentieth annual meeting during October, 1974, and this Association and other public health organizations and the affected industries were represented and participated in AMHIC's discussion.

Vending machine evaluation program administrative policies

The members of AMHIC after thoroughly reviewing several significant proposed revisions to the NAMA Vending Machine Evaluation Program Administrative Policies approved the proposals, and a copy of these revised policies may be obtained from NAMA.

NAMA-AMHIC Organization plan and procedures

The members of this Committee, as with the Administrative Policies, submitted comments on the proposed revisions to the NAMA-AMHIC Organization Plan and Procedures before any action by AMHIC. Then, AMHIC after making minor modifications to the preamble, voting procedures, and the Nolan Award Criteria approved the proposed revisions to the Organization Plan and Procedure. Copies of this document may also be obtained from NAMA.

Ice makers for vending machines

The proposed protocol of a Study of Ice Maker Automatic Cleaning Systems is being finalized. Furthermore, two manufacturers have agreed to provide test machines; and the studies using this protocol to

determine the effectiveness of automated ice maker cleaning systems will be conducted near the NAMA headquarters for the purpose of convenience and adequate control. The following items are contained in the protocol: purpose of the study, models of ice making cleaning equipment to be studied, procedures for testing equipment, sampling and laboratory procedures, and reporting results.

It was also recommended by the Public Health representatives that the proposed study of ice maker automatic cleaning systems be extended to include large volume ice maker dispensers designed for institutional and hotel/motel ice. However, it was reported by a member of the ice industry that such machines at present are not currently equipped with automatic cleaning systems.

In addition, there was a discussion of the recently developed machines which manufacture and dispense bagged ice, manually or by coin operation, and the possible need to augment the NAMA ice maker study to cover such equipment.

Two other proposals are being considered by the manufacturers of ice making equipment for vending machines, one would involve the use of a chemical to soak, delime, and sanitize the food contact surfaces and the other one would involve a different type of chemical to keep bacterial populations at a low point. These newer processes could result in a revision of the Ice Maker Study Protocol.

Revision of the NAMA evaluation manual

The following additional item (904) entitled *Machine Model Identification* was approved by the members of AMHIC for inclusion in the Evaluation Manual: For the convenience of inspection and service personnel, the manufacturer shall post his company name and the machine model designation: (*a*) on the cabinet front; or, (*b*) inside the display area visible through the front; or, (*c*) prominently inside the cabinet. However, this requirement shall not be applied to small machines, such as ballgum and table-top venders, whose data plate can readily be seen under normal location conditions.

Carbonation backflow

The problem of carbonation backflow has not been resolved by requiring vented check valves in directly-connected dispensers even with all copper removed from the vending machines. The vending industry has made extensive and expensive studies (one company has spent nearly $100,000 in developing valves to prevent carbonation backflow) on vented valves without resolving the potential problem of carbonation backflow and the resulting possibility of copper poisoning. During this discussion, representatives of NAMA and NSF were present and assured the members of AMHIC that the final resolution of this matter would be applied uniformly by both agencies as it related to coin operated vending machines.

According to the NAMA Staff, the following options available to improve backflow preventions are not unlimited and have been discussed in previous reports.

(*a*) Require a mandatory air gap on all post-mix units, coin-operated (and manual). With internal stainless steel tubing (which these new units have), the system would be 100% fail-safe.

However, an air gap means a reservoir with floats or electrodes. In this or office-type machines serviced by non-trained employees or in a manual dispenser, the sanitary maintenance of a non-pressurized water container could be a step backward in public health protection.

(*b*) Allow directly-connected units to employ the traditional USPHS Vending Code safeguards but rule out all internal copper water tubing.

In cases of short-term check valve failure, it is possible that backflow will not penetrate the upstream water tubing beyond the 20+ ft of tubing in the pre-cooling coils. On the other hand, there are reported cases of backflow entering water supply piping systems outside the equipment.

The exclusive use of stainless tubing internally could well provide improved protection. It would be incorrect to assume, however, that 100% protection could be guaranteed except at locations where the external, inhouse supply lines were not copper.

(*c*) Retain the present Vending-Code backflow requirements and, when later technology permits, upgrade the NAMA and NSF standards to require whatever improved protection can be attained.

PHS/FDA vending code

The members of AMHIC agreed that an ad hoc committee would be appointed, and this committee should prepare a statement on proposed Code revisions at the earliest possible date in the form of an AMHIC initiated request to FDA for a revision of the 1965 Vending Code. Further, this committee would address itself specifically to the question of carbonation backflow in directly-connected dispensers, limited service vending machines (special dispensers), and the first draft of a set of proposed Code revisions prepared by the NAMA Staff. The ad hoc committee met in February and prepared a set of recommendations for revising the Code, which NAMA sent to FDA. It is believed that if these proposals are accepted by FDA that the current problems with special dispensers and carbonation backflow will, for the most part, be resolved; and the Code will be more easily understood and applied by both public health and industry.

Vinyl chlorine study

Some members of AMHIC recommended that NAMA initiate a study of the extent of migration of vinyl chloride monomer from PVC tubing and other contact parts used in vending machines, unless such data are available from present sources. The NAMA Staff reported previous NAMA surveys and contacts with FDA and the Society for the Plastic Industry (SPI) and that specific studies would be initiated by NAMA should be proprosed SPI Report to FDA fail to cover all vending usages of PVC

Food shelflife

NAMA has decided to assist its members in re-evaluating the vending industry's long-time practices in discarding "over-age" foods returned from perishable food and pastry vending machines. This decision was based in part on the fact that food should not be wasted at a time when food shortages and famine are found to be of worldwide proportions.

NAMA has made plans to: (*a*) survey typical vending companies to determine present shelflife practices by product category; (*b*) invite all AMHIC members and observers to provide suggestions and/or data on any known shelflife studies which concern commissary-type products and pastries; and (*c*) have conducted by a NAMA consultant agency a sampling and quality study of commissary foods to determine whether shelflife guideline development is practicable. This Committee will be expected to comment on these proposals within the next year.

RECOMMENDATIONS

(*a*) The Association reaffirms its support of the National Sanitation Foundation and the National Automatic Merchandising Association and continues to work with these two organizations in developing acceptable standards and educational materials for the food industry and public health.

(*b*) The Association urges all sanitarians to obtain a complete set of the National Sanitation Foundation's Food Equipment Standards and Criteria and a copy of the National Automatic Merchandising Association—Automatic Merchandising Health-Industry Council's Vending Machine Evaluation Manual and related educational materials; to evaluate each piece of food equipment and vending machine in the field to determine compliance with the applicable sanitation guidelines (construction and installation specifications); and to let this Committee and the appropriate evaluation agency know of any listed manufacturer or fabricator failing to comply with these guidelines.

(*c*) The Association urges all sanitarians and regulatory agencies to support the work of the Association's Committee, submit suggestions for developing new guidelines and for amending same, and subscribe, by law or administrative policy, to the principles represented by the Standards, Criteria, and Evaluation Manual for Food Equipment and Vending Machines.

This report of the IAMFES Committee on Food Equipment Sanitary Standards is respectfully submitted by:

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News and Events

ISI® Creates New Educational Position

The Institute for Scientific Information® (ISI®) has created a new position — Lecturer-Professional Education—designed to help academic institutions enhance their instructional programs on the use of scientific and technical information.

Starting in 1976, ISI® will offer without charge a professional reference librarian/educator to serve as a guest lecturer, seminar leader, or panelist who will provide instruction on the role the company's services play in information retrieval for the sciences and the social sciences. Participation by the ISI® lecturer will be by invitation from the schools and presentations will be tailored to meet the needs of graduate or undergraduate students.

ISI® sees two major sources of demand for its new program. One is library and information science shools which can use the lecturer to provide specialized instruction to prospective librarians on the ISI® services they will need to know about when they assume professional positions. The other is the students and educators in the sciences and social sciences who would profit from a better understanding of how information services can help them with their work.

The first person to fill the new position is Diane J. Hoffman, a Syracuse University graduate with seven years' experience as a reference librarian.

Requests for additional information or reservations for lectures should be addressed to Lecturer-Professional Education, Institute for Scientific Information, 325 Chestnut Street, Philadelphia, Pa. 19106. Phone (215) 923-3300.

Dairy Laboratory Workshops

Two Dairy Laboratory Workshops have been scheduled at The Pennsylvania State University during the summer of 1976. Workshop I is scheduled for June 21-25, and Workshop II for June 28-July 16. Workshop I will cover composition and properties of milk, testing dairy products for fat and total solids, milk cryoscopy, acidity testing of dairy products, analysis of cleaners and sanitizers and organoleptic evaluation of dairy products. Workshop II will cover the microbiological and chemical procedures required for certification as a Pennsylvania Approved Dairy Laboratory Director.

For further information, please contact George H. Watrous, Jr., 107 Borland Laboratory, University Park, PA 16802.

USDA Announces Membership for New Salmonella Advisory Board

The U.S. Department of Agriculture (USDA) has announced selection of 13 representatives to serve on the recently created Advisory Committee on Salmonella.

The committee objective, according to USDA's Animal and Plant Health Inspection Service (APHIS), is to reduce salmonella contamination of meat and poultry.

The committee chairman is Richard L. Feltner, assistant secretary for marketing and consumer services. Vice Chairman is Dr. Francis J. Mulhern, administrator of APHIS. Executive Secretary is William H. Dubbert, chief, Systems Development and Sanitation Staff, Meat and Poultry Inspection Program, APHIS.

The duties of the advisory committee are to advise the Secretary of means to limit the spread of salmonella contamination during slaughtering, eviscerating and further processing operations; to recommend and solicit the cooperation of affected industries in implementing measures which are developed; to recommend regulatory requirements needed to apply critical control procedures; and to consider means of disseminating information on preventative practices to industry and to consumers.

Western Food Industry Conference Schedule

The 5th Annual Western Food Industry Conference, which is co-sponsored by the Northern and Southern California IFT Sections, the California Dairy Industries Association, and the University of California, will be held at the University of California at Davis on March 30, 31, and April 1, 1976. *Looking Ahead Through Education* is the theme of this year's 2½ day conference.

The conference's program is designed to update and further the technical and scientific expertise of the Western food processors. The schedule of meetings will include two general sessions and several concurrent sessions. Conference attendees are encouraged to alternate between the concurrent sessions in order to best suit their professional interests.

Beginning on Tuesday morning, the conference will open with timely topices affecting California's food industries, the *Changing Role of the West in Agriculture; Issues Facing the California Department of Food and Agriculture;* and, *Current Criticism of the Food Industry and How to Respond.* In the afternoon three concurrent sessions on *Energy Use, Conservation and Alternatives in Food Processing; Processing and Marketing Loss Reduction;* and *Dairy Product and Processing Concerns* will be presented.

Three concurrent sessions will head Wednesday morning's prgram. Disposal concerns of the food industry will be covered in *Waste Management;* future profits, supply and processing, regulatory trends, and consumer trends are topics that will be discussed in the session on *Trends in the Dairy Industry;* and, techniques for evaluating nutritional composition will be mentioned in the *Analytical Systems for Nutrients* session.

Wednesday afternoon will also consist of three concurrent meetings. There will be a continuation of the morning's session on *Trends in the Dairy Industry.* Recent developments in processing will be reviewed at the session, *What's New in Processing.* The third session will feature current topics related to *Nutritional Labeling.*

The closing general session of the conference on Thursday morning will be directed at *Sensory Evaluation.*

For registration information and other details, contact: John C. Bruhn, Extension Food Technologist, Food Science & Technology Dept., University of California, Davis, CA 95616. Phone: (916) 752-2192.

NACTA Meets in Lubbock

The National Association of Colleges and Teachers of Agriculture will hold its annual meeting June 16-18, 1976. It will be held on the campus of Texas Tech University in Lubbock, Texas. The annual meeting will feature presentations on effectiveness in teaching, trends in agricultural curricula, and items of interest to teachers of agriculture.

For information on the meetings, write Dr. J. Wayland Bennett, Box 4190, Texas Tech University, Lubbock, TX, 79409.

Kultures and Kurds Klinic Set

The 1976 American Cultured Dairy Products Institute Training School will be held at the Hospitality House Motor Inn, Crystal City, Virginia on March 29-31, according to Dr. C. Bronson Lane, Secretary for the Institute.

Purpose for the "Klinic" is to update cultured products plant personnel and allied tradesmen on the newest quality control programs, processing procedures, culture technology, and marketing techniques.

Buttermilk, sour creams, yogurts, and cottage cheese brought by the conferees will be evaluated by experts. Individual awards will be given to manufacturers producing superior quality cultured commodities—with the over-all winner taking home the prestigious Neil C. Angevine Trophy presently held by T. G. Lee Foods, Orlando, Florida.

A tour of the Safeway and Giant foods processing plants in the D.C. area is also on tap for Klinic delegates.

For additional information and for advance registration forms, contact John Speer, ACDPI Treasurer, 910-17th Street, N.W., Washington, D.C. 20006.

Cleans Plant Waste Stream and Saves $17,000 a Year

A four-page illustrated bulletin showing how one company saves $17,000 a year by using a UCARSEP® Ultrafiltration System to cleanse its waste streams is now available from Union Carbide Corporation.

The company, a manufacturer of coated metal coil, had been dumping oil, dirt, and phosphate-rich detergent into its municipal waste system. A new state law said "No," and the bulletin outlines what happened. It tells of solutions that were tried and discarded, and it describes the UCARSEP System that proved to be the answer: eliminating municipal sewer charges formerly paid, eliminating the costs of former cleanups; recovering valuable washer chemiccals; eliminating the need to pre-cool bath streams for treatment; and improving the company's end product.

The UCARSEP Ultrafiltration System removes 100 per cent of suspended solids from the waste water. It separates oil-water emulsions without the use of emulsion-breaking chemicals. It separates colloids and polymers down to 50 angstrom units in diameter.

A key to its success is an inorganic membrane which easily withstands strong caustic solutions (even at boiling point). In addition, it cleans easily for continued use.

Copies of the bulletin, "Processing Metal Cleaner Bath Solutions with UCARSEP® Ultrafiltration Systems," are available from Dept. WSY, 100 Clearbrook, Union Carbide Corporation, P.O. Box 65, Tarrytown, NY. 10591, or call 914-345-2659.

Book Review

Nutrition Technology of Processed Foods, Nicholas D. Pintauro Published by Noyes Data Corporation, Park Ridge, New Jersey and London, England, 1975, 332 pp., $36.00.

This book is a valuable reference for sanitarians and for the many people that are concerned with processed foods. Timely discussion is given on processed food ingredients and their nutritional value. Areas such as cholestrol and dietary concerns are covered. There is valuable information for food processors, sanitarians, and fieldmen as well as for students in colleges and universities. The book asks the food technologist to think of enrichment in terms much more complex than the simple addition of vitamins, minerals, and proteins. Practical recommendations are here for helping to form a new American nutritional pattern that is now emerging because of economic conditions, a desire to reduce carbohydrate intake, to eat less saturated fat and cholesterol containing foods and to reduce total lipids and caloric intake in general.

Nutrition Technology is well organized and very readable. Several subject indices enable the reader to have easy access to all of the information in the book. In addition, other indices by company, inventor and patent number are supplied in the back of the book to give further help in providing quick access to specific information.

The detailed, descriptive information is based on U.S. patents that deal with the nutrition technology of processed foods. This book serves a double purpose in that it supplies detailed technical information and can be used as a guide to the U.S. patent literature in this field. It deals with the food and nutritional aspects of nutrition technology of processed foods based on United States patents. It would serve as an excellent reference to people working in food safety fields.

Supplemental ingredients and the necessary technology to produce products that are especially designed for improvement of human health are stressed in the book.

Practical recommendations to the food industry are presented. Based on the most recent U.S. patents, 127 food manufacturing processes are described. Chapter headings are given. Numbers in parenthesis indicate a plurality of processes per topic. 1. Cereal Products (11), 2. Enriched & Dietitic Breads (12), 3. Rice and Potato Products (15), 4. Milk and Dairy Products (10), 5. Nutritious Beverages (16), 6. Low Cholesterol Egg Products (7), 7. Salt Substitution (8), 8. Complete Diets (14), 9. Special Fortification Processes (12, and 10. Special Processes & Products (*19*).

Earl O. Wright
Executive Secretary
I.A.M.F.E.S.
Ames, Iowa 50010

Food Scientist Honored

Prof. Robert R. Zall, of the New York State College of Agriculture and Life Sciences, Cornell University, has been presented a Certificate of Appreciation by the regional office of the U.S. Environmental Protection Agency.

The citation recognizes Zall's "contributions to a better environment."

Zall, a member of the College's Department of Food Science, was specifically noted for his efforts to help the dairy industry deal with their environmental pollution problems.

He has designed systems to deal with cheese whey products, recycle food plant wastes into usable food ingredients, reuse hot water and cleaning solutions and treat milking center waste effluents in lagoons. On campus, he also teaches a course in food sanitation.

New National Nutrition Consortium Communications Guidelines for Nutrition Labeling

"Nutrition Labeling—How it Can Work for You," a 134-page, two-color booklet intended for anyone involved in communicating the meaning of the information on nutrition labels, is now available.

The Consortium brought together experts to produce the basic text on nutrition and how information on nutrition labels can be used in nutrition education and meal planning. Ronald M. Deutsch, author and lecturer, was retained to write the final version.

Profusely illustrated with charts, tables and drawings, the publication is divided into two sections: a nutrition primer describing the functions and dietary role of each of the basic nutrients—protein, carbohydrates and fats, vitamins and minerals—and a parallel section describing methods by which lable information on each nutrient can be used in menu selection. How label information can be used in meal planning to meet special dietary and economic needs is discussed.

There is discussion of the meaning of the "U.S. RDA," label terminology in formulated foods, the role of food additives, and a section on still-unresolved problems related to food processing and marketing.

The booklet is being distributed on a complimentary basis to food editors of the mass media, ad agencies, copy writers, nutrition educators, extension agents, physicians and others who discuss nutrition with the public. Single copies are available at a cost of $2.00 each from: Nutrition Labeling, P.O. Box 4110, Kankakee, Illinois 60901. Lower cost bulk rates are available upon request.

Maryland Dairy Shrine Honors Extension Men

DAIRY SHRINE CONTEMPORARY—Dr. and Mrs. Wendell S. Arbuckle of College Park and Dr. Richard F. Davis of Adelphi (left to right) pose with Dr. Arbuckle's portrait following its official induction into the Maryland Dairy Shrine hall of fame during a mid-October bull stud open house north of Frederick. All are Prince Georges county residents. Dr. Arbuckle served as a College of Agriculture faculty member, research worker and Extension dairy products specialist at the University of Maryland from 1949 until his retirement in 1972. He has national and international stature in the field of ice cream manufacturing. Dr. Davis is dairy science department chairman at the College Park campus. He made the recognition presentation honoring Dr. Arbuckle.

PMA Releases Compilation of Polyurethane Testing Standards

A compilation of the more significant ASTM testing standards applicable to the design and specification of polyurethane products has been recently released by the Polyurethane Manufacturers Association. The compilation was made of testing standards covering not only polyurethane, but also those which can be adapted for use with both rubber and polyurethane, according to Jack Schreiber, of Rubber & Silicone Products Co., who is Chairman of the PMA Standards Committee.

Twenty-four testing methods or standards are included in the 214-page book plus the ASTM classification system for elastomeric materials for automotive applications. The information in the book should be of use to designers, processors, and specifiers of a wide range of polyurethane products including solid cast wheels, rollers, coating, and sealing devices. Data related to durability and to the effect of heat and cold on various types of polyurethane is included.

Copies of the compilation are available from PMA Headquarters at 180 North Michigan, Chicago, Ill. 60601 at $15.00 per copy to non-members.

Heldman Appointed MSU Department Chairman

Dr. Dennis R. Heldman has been named chairman of the Department of Agricultural Engineering at Michigan State University.

He succeeds Dr. Bill A. Stout, who remains in the department as a professor, specializing in worldwide use of energy for agriculture.

Heldman was professor within the department specializing in food engineering. He joined MSU in 1965 as instructor after receiving his Ph.D. from MSU. He holds B.S. and M.S. degrees from Ohio State University.

He recently completed a year in the Academic Administration Internship Program of the American Council on Education. The program places 40 university faculty from around the country in university central administrations. His internship was completed at Ohio State in the Office of the Provost.

Heldman edited the Food Engineering Newsletter of the American Society of Agricultural Engineers (ASAE) from 1968-70 and was chairman of the DFISA-ASAE Food Engineering Award Committee. Within the Food Engineering Division, he served on a number of committees including: nomenclature, education, program, publications and food processing. Currently he is director of this division and a member of the board of directors of the society.

He was chairman of the Food Engineering Division in 1970-71. He has served as the Michigan section secretary-treasurer and as vice chairman.

He is the author or coauthor of over 120 technical research papers either published or presented at national meetings between 1963 and 1975.

During this time he received three American Society of Agricultural Engineers Paper Awards for outstanding presentations. His textbook, "Food Process Engineering," published in 1975, is being used in food engineering courses at most universities.

Heldman is a member of the American Society of Agricultural Engineers, Institute of Food Technologists, American Dairy Science Association, American Society for Engineering Education, Society of Sigma Xi, Phi Kappa Phi, Alpha Epsilon and Phi Tau Sigma. He has served on a number of national committees of the first three organizations.

He was awarded the Young Engineering Achievement Award for Research by the American Society of Agricultural Engineers at the annual winter meeting in Chicago on December 11, 1974. The award is presented annually to the outstanding agricultural engineering researcher under 40.

Acknowledgment of Assistance by Reviewers

Appreciation is expressed to all members of the Editorial Board who reviewed manuscripts during 1975. Thanks also goes to Dr. T. E. Minor, Department of Preventive Medicine, University of Wisconsin-Madison, who assumed editorial duties for 6 weeks during 1975 while the Editor was in Europe.

During 1975 manuscripts were reviewed by the following persons who were not regular members of the Editorial Board. Their help is acknowledged and appreciated.

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ELMER H. MARTH
Editor
Journal of Milk and Food Technology

Letter to the Editor

Ineffective food protection

DEAR SIR:

The Comptroller General of the United States (GAO) submitted to the Congress on December 8, 1975 a report, *Federal Support for Restaurant Sanitation Found Largely Ineffective.* The report was well publicized in the daily press and several of the nation's leading newspapers published favorable editorial comments on the findings and conclusions of GAO. This was not the first report to point up deficiencies in food protection services at all levels of government.

Food protection has increasingly been the keynote or theme of local, state and national professional meetings and discussion. These discussions, like the GAO report, corroborate the need for a more comprehensive and effective approach to food protection, including both aesthetics and public health dimensions.

In reviewing the report one could at the outset, criticize the statistical approach and some aspects of the survey methodology. But it would be ungenerous if not unwarranted to cavil at the author's efforts. Instead it would be more productive to suggest an approach for improving the capacity and capability of regulatory agencies to provide maximum food protection services.

A starting point may well be development of a national certification or accreditation program for food protection services at the state and local levels. The objective would be to establish standards of quality for food control programs through a process of evaluation, education, and consultation. This would encourage and assist local officials (health and political decisionmakers) to meet and maintain standards and improve the full range of resources required for effective food protection. It would also provide an appropriate frame of reference for ordering food protection among other city government concerns such as police and fire services, public works, recreation, and social services.

A basic function of standards irrespective of the standard-setting program is to serve as guidelines and indicators. In the first instance the written standards provide guidelines to the responsible food protection official indicating the general criteria to be met to achieve the goal of certification or accreditation. And in the second instance, when standards are applied they serve as indicator to the standard-setting organization, which may be FDA, that when the state or local food protection agency complies with the standards it has demonstrated a capacity to provide a stated level of food protection. The key element here is that standards define a certain capacity for a quality food protection service and not the quality itself.

We assume that given this capacity a level of quality of food protection will result. And experience informs us that without this capacity (which includes a full complement of resources and supporting services), achievement of quality is difficult if not impossible.

BAILUS WALKER, Jr. .
Department of Environmental Services
Environmental Health Administration
Government of the District of Columbia
Washington, D.C. 20002

Crumbine Consumer Protection Award Open to Entries from Local Agencies

Entries are now being accepted for the 1976 Samuel J. Crumbine Consumer Protection Award, given annually by the Single Service Institute to honor outstanding achievement by a local government in developing a program of food and beverage sanitation.

Contestants for the 1976 prize are being asked to demonstrate specifically the use of effective planning and management techniques by officials of local government agencies in the development of comprehensive food protection programs.

The winning government agency or department receives a bronze medal and engraved plate mounted on a walnut plaque. The presentation will be made at the annual convention of the International Association Of Milk, Food and Environmental Sanitarians, August 11, 1976, at Arlington, Illinois.

Entries must be submitted on or before May 31, 1976, to The Crumbine Jury, c/o Environment & Health Committee, Single Service Institute, Inc., 250 Park Avenue, New York, N.Y., 10017. The official Award announcement, containing full instructions, may be obtained by writing to the above address.

The Institute is the national trade association of manufacturers of single-use food service and packaging products. The Award honors the late Dr. Samuel J. Crumbine, longtime Health Officer for the State of Kansas and a pioneer in public health programs.

The 1976 Award focuses on effective planning and management at a time when tax funds are severely limited and "must cover a broad spectrum of competing government activities, of which food protection is only one." Increasingly, the announcement points out, "local public health agencies must earn their way through the use of the best techniques of planning and management."

The 1976 Samuel J. Crumbine Consumer Protection Award aims specifically "to encourage and recognize food protection programs based on these techniques."

The Award Jury is made up of consumer representatives and leading professionals in the public health and environmental fields.

63RD ANNUAL IAMFES
1976 STAR SPANGLED MEETING
ARLINGTON PARK HILTON
ARLINGTON HEIGHTS, ILLINOIS
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Association Affairs

Report of voting on Amendments to the Constitution:

1. Amend Article IV, Section 1 by striking out:
   (a) "and" in lines 4 and 5
   (b) "second vice-president and" from lines 5 and 6 by adding:
      (i) "and Secretary-Treasurer" following vice-president in line 4.
      (ii) "and second vice-president" following first vice-president in line 5.

   VOTE: Yes—339
   No — 23
2. Amend Article VII, Section 1 by substituting for the words "Secretary-Treasurer" the words "Executive Secretary."

   VOTE Yes—356
   No — 5

Affiliate Annual Meetings

California—October 20-22, 1975, Queensway Hilton, Long Beach.
Connecticut—January 1976.
Florida—March 16-18, 1976, Langford Hotel, Winter-Park.
Illinois—December 1, 1975, Blue Moon Restaurant, Elgin.
Indiana—October 7-9, 1975, Holiday Inn, Merrillville.
Iowa—March 22, 1976, Ramada Inn, Ames.
Kansas—October 1-3, 1975, Holiday Inn, Manhattan.
Kentucky—February 24-25, 1976, Stouffer's Inn, Louisville.
Michigan—March 1976.
New York—September 17-19, 1975, Granit Hotel, Kerhonkson.
Ontario—Eastern, November 1975, Kemptville.
Oregon—November 17, 1975, Oregon Department of Agriculture & Kings Table of International Restaurant, Salem.
South Dakota—May 11-14, 1976, Holiday Inn, Aberdeen.
Virginia—March 8-10, 1976, Donaldson, Brown Center, Blacksburg.
Washington—September 9, 1975, Sheraton-Renton Inn, Renton.
Wisconsin—September 25-26, 1975, Holiday Inn, Tomah, Wisconsin.

Illinois Affiliate Hosts Fall Conference

Robert Coe, Secretary-Treasurer (Front Right); Dr. George Muck, President (Front Left); Charles Price, President Elect (Rear Left); Lewis Schultz, First Vice President (Rear Center); John Oberweiss, Second Vice President (Rear Right).

The fall conference of the Associated Illinois Milk, Food and Environmental Sanitarians, Inc., was held December 1, 1975, at Elgin, Illinois. Ninety sanitarians and guests were in attendance.

The day long program included presentations on many subjects of interest including "Registration of Pesticides," "Canned Food Microbiology," "The Chemistry of Cleaning," and "Nutritional Labeling." A timely paper on sampling surveillance of milk and milk products was presented by Ray Belknap and Mr. Robert Van Liere, General Manager of AMPI discussed some of the current trends in milk marketing.

The C. B. Shogren Award, presented to the Illinois Affiliate at the 1975 International Meeting in Toronto, was presented to the members by affiliate representative Bob Coe and Affiliate President George Muck.

During the business meeting a report on the 62nd annual meeting in Toronto was made by IAMFES Executive Secretary Earl O. Wright. Charles Price gave a progress report on the status of local arrangements for the 1976 Annual Meeting which will be hosted by the Illinois Affiliate at the Arlington Park Hilton Hotel, Arlington Heights, Illinois, on August 8-12, 1976. In support of hosting the 1976 meeting more than 20 new members have been signed up.

Under the direction of the Executive Board the Illinois Affiliate is re-establishing the P. E. Riley Award. This award, given in past years, is presented to an outstanding sanitarian for contributions to improvement of public health in the State of Illinois. Nominations are being solicited for the next presentation which will be made during the Illinois Affiliate luncheon to be held during the 1976 IAMFES Annual Meeting.

CLASSIFIED ADS

The State Laboratories Department in Bismarck, North Dakota, has a position open for a supervisory sanitarian. The responsibility accompanying this position involves supervision of a Statewide food service sanitation program plus management of a Statewide program involving sanitary inspections of hotels, motels and trailer courts.

Inquiries may be directed to Robert J. Olheiser, State Food Commissioner and Chemist, State Laboratories Department, Box 937, Bismarck, North Dakota, 58505. (701-224-2486).

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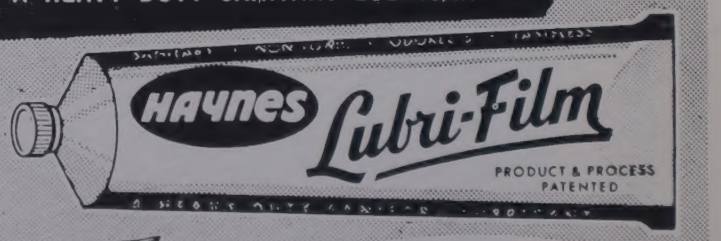

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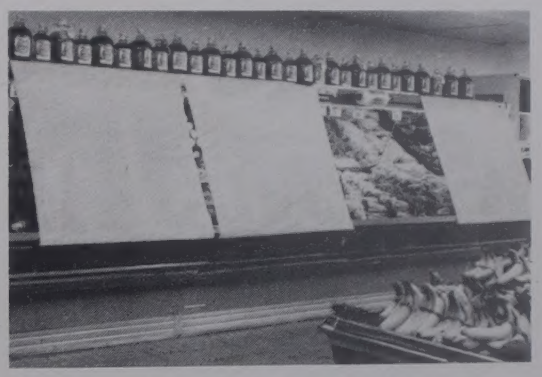

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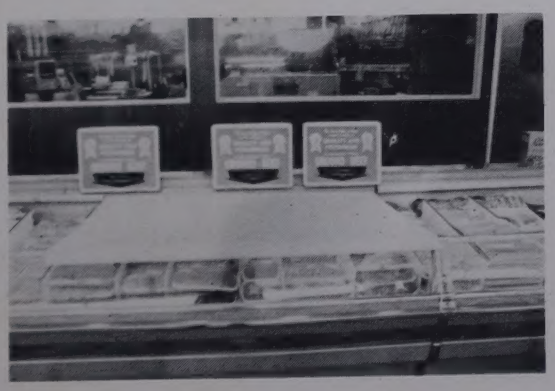

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Dairy authorities speak out on better cow milking

Stephen B. Spencer
Extension Dairy Specialist,
Pennsylvania State University

What's your score on vacuum?

An inadequate vacuum pump can affect the operation of the milking units drastically. Excessive "drop-off," slower milking, incomplete milking and an increase in the incidence of mastitis are likely results.

As a dairyman, your net dollar income depends on the vacuum pump and it probably is used more hours each year than most farm tractors.

Few people would hitch six plows behind a four plow tractor and head for the field to do a day's work. Yet many of these same people milk cows with a short vacuum supply and never question whether the pump is adequate.

Vacuum pumps used to be rated according to the number of units that could be operated. Today we measure the pump capacity in terms of Cubic Feet of air per Minute (CFM). Just as "horsepower" is more reliable than "plow rating" as an indication of tractor size, "CFM" is more reliable than "unit rating" when sizing a vacuum pump.

CFM output depends upon vacuum level. As vacuum level increases, the CFM output decreases. It's important to operate the system at the vacuum level specified by the manufacturer or the output of the pump will be altered.

The important consideration of any vacuum pump is the CFM output at the milking vacuum level. The pump must have adequate CFM output to meet the vacuum requirements of the system and provide sufficient reserve to maintain a constant vacuum level.

Vacuum Requirement
The milking unit is the most important of the machine components which admit air into the system. The air consumption of milking units varies depending upon shell and inflation size, pulsation rate and length and size of pulsated air tubes. Typically, the air requirement of a milking unit while it is not milking is three to four CFM. The pulsator consumes 50 to 70% of this volume. Considerably larger air pumping capacity (a reserve) must be provided to make a milking system operational. Other components which consume air are such things as vacuum operated door openers, milk metering devices, and the vacuum regulator. The requirements of each component must be added together to determine the system requirements.

Vacuum Reserve
The vacuum reserve is the air pumping capacity which remains after the vacuum requirement of all components has been satisfied. That's the problem. We've thought in terms of the vacuum reserve as the amount of CFM capacity that's *left over*. We really should be thinking in terms of a *base reserve* for the operator(s) before we begin to compute the system requirements.

The reserve is all-important in order to maintain vacuum stability. The reserve is necessary in order to make allowances for operator usage and possible leaks in the system or other contingencies.

The most important reason for an adequate vacuum reserve is to provide for the amount of air that the operator will use. The operator is the largest user of the vacuum reserve. Some operators are very wasteful of the available reserve. This occurs as units are being attached and removed. Improper unit adjustment is also a significant factor. When teat cups start to leak and "squeal" during milking, the vacuum reserve is depleted rapidly.

Some operators may deplete vacuum reserve as much as 30 or more CFM for short periods of time. The careful operator will use but half that amount during the milking process. The real test of any milking system is when a milking unit falls off. It takes huge reserves of air just to keep the remaining units on the cows. Reserve tanks aid a little during these occurrences but basically the vacuum pump must be relied upon to maintain vacuum level. It all adds up to the fact that an adequate pump is a *must* for every dairyman.

Research in Ireland, Wisconsin, Pennsylvania and California indicates that inadequate vacuum reserve is associated with higher leucocyte counts. In plain language it means that mastitis can result if your vacuum pump isn't large enough.

What's your Vacuum Score?
Don't make a mistake and just assume that your pump is putting out enough air. Have it checked with an air flow meter once a year. Many dealers are equipped to do this for you.

And how will you know for sure that they're not just trying to sell you a pump? Frankly, I've found most dealers to be very reliable in this respect.

There have been many different recommendations about pump sizes. It's hard to give one that's exactly right for each system. Here's a guide for you to check your vacuum needs. It's based upon the New Zealand Standard. The American Standard would give values equal to one-half of the New Zealand Standard.

For bucket users:
Allow 4 CFM per unit + 20 CFM base reserve.

For pipeline users:
Allow 5 CFM per unit + 40 CFM base reserve for the first operator and 20 CFM for each additional operator.

The resulting CFM values would give you the minimum size vacuum pump capacity. If your system has more than this, fine. If you have less vacuum capacity than this you should carefully investigate your vacuum needs.

This method of determining vacuum capacity is different from what you may have seen before. A 50 percent reserve is commonly used. While a 50 percent reserve may be satisfactory on a system of six or more units, our field studies indicate that using a 50 percent reserve is not adequate for the smaller system.

Remember this: There is no substitute for an adequate vacuum system. Make sure you know your score on your vacuum needs.

This is one of a series of topics developed by noted Dairy authorities. For a complete set write for a free booklet.